Dye-sensitized photolability of the Escherichia coli ribosome
by Robert Thomas Garvin

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Chemistry
Montana State University

Abstract:
Chemical modification of the Escherichia coli ribosome by dye-sensitized photooxidation has been shown to be very effective in reducing in vitro amino acid incorporation.

This effect can probably be ascribed to photochemically-mediated oxidation of specific surface protein(s) for the following reasons: (1) functional relationships between tRNA, mRNA, and the ribosome are more likely confined to the surface of the ribosome owing to the bulk of the constituents; (2) the dyes used as sensitizers do not normally have access to internal regions of protein structures; and (3) a comparison of dyes efficient for sensitizing the photooxidation of either guanine in RNA or amino acid residues in proteins revealed that a protein (or proteins) were the most likely photolabile centers.

Synthetic messenger (C^14-polyuridylic acid) binding decreased with time of photooxidation. Preincubation of the ribosome with polyuridylic acid afforded complete protection against inactivation by dye-sensitized photooxidation.

The ribosomal subunits were separately photooxidized, recombined with their un-photooxidized native complementary particle, and the in vitro incorporation capacity of the photooxidized/native complex was assayed. The 30S subunit was much more sensitive to dye-sensitized photooxidation than the 50S subunit.

A preparation was devised for the isolation of bacterial ribosomes using diethylaminoethyl-cellulose with ammonium chloride washing and elution.

Small-scale methods for assaying the biological activity of bacterial ribosomes in vitro were developed for the polyadenylic- and polyuridylic acid-directed systems.

The effect upon ribosomes of certain thiacyanines was shown to be a dark inactivation of their biological activity.

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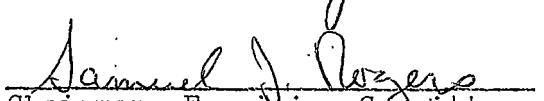

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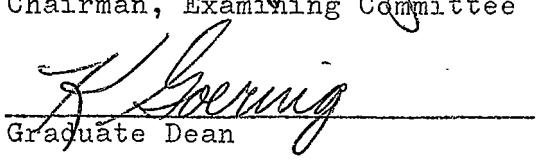

Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

August, 1969

ACKNOWLEDGMENTS

The author thanks:

CHARLAND ROSS GARVIN, especially, for her continued emotional support during the course of this, and other, studies;

DR. SAMUEL JOHN ROGERS for his creative guidance, constructive criticism, and personal and intellectual involvment throughout this investigation;

and DR. GORDON RAY JULIAN, whose warmth, interest, intellectual attainment, and personal expertise provided the example of humanitarian investigator.

This work was supported, in part, by National Institutes of Health Pre-Doctoral Fellowship 1-F01-GM41409-01-BNB.

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ABSTRACT

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This effect can probably be ascribed to photochemically-mediated oxidation of specific surface protein(s) for the following reasons: (1) functional relationships between tRNA, mRNA, and the ribosome are more likely confined to the surface of the ribosome owing to the bulk of the constituents; (2) the dyes used as sensitizers do not normally have access to internal regions of protein structures; and (3) a comparison of dyes efficient for sensitizing the photooxidation of either guanine in RNA or amino acid residues in proteins revealed that a protein (or proteins) were the most likely photolabile centers.

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INTRODUCTION

" . . . the secrets of nature betray themselves more readily when tormented by art than when left to their own course."

Francis Bacon, Novum Organum

The first studies on *in vivo* incorporation of radioactive amino acids into proteins were made on chicks (Hultin, 1950), mice (Borsook, 1950), and rats (Keller, 1951). When rat liver fractions were tested for their ability to incorporate C^{14}-alanine *in vitro*, the highest specific activity was associated with the microsomes (Siekevitz, 1952) -- a term coined by Claude to denote a cellular fraction sedimenting in a centrifugal field under certain well-defined conditions (Claude, 1946). When this microsomal fraction was disintegrated by a detergent, the radioactivity was found in the "detergent-insoluble" portion, which was shown by ultracentrifugational studies and electron microscopy to consist chiefly of particulate ribonucleoproteins of molecular weight 2.7 - 4.5 million daltons, containing 50 - 70% ribonucleic acid (RNA), with no lipid (Littlefield, 1955). These particles were called ribosomes by Roberts (Roberts, 1958). Proof that the ribosome was the cellular component responsible for protein synthesis was provided in 1957 by Littlefield and Keller, who showed that ribosomes from mouse tumors, separated from their microsomal membranes, incorporated amino acids *in vitro*.

It has since been shown possible to remove the bacterial ribosome from its natural environment, supply it with the necessary protein factors, energy sources, and exogenous

message, and have the ribosome function in a more or less accurate fashion *in vitro*, thus copying, superficially at least, its action *in vivo*.

The discovery made by Nirenberg and Matthaei (Nirenberg, 1961) that polyuridylic acid (poly U) acted as the message for polyphenylalanine formation greatly simplified the study of protein synthesis, since poly U could be synthesized by ordinary chemical methods. The essential components for the poly U-directed synthesis of polyphenylalanine *in vitro* are: ribosomes; proteins (enzymes and "factors") isolated from the cellular supernatant fraction; adaptor molecules called transfer RNA's (tRNA); the ions Mg^{++}, and NH_4^+ or K^+; and GTP. Table I summarizes the factors that have been identified as necessary for protein synthesis in studies subsequent to those of Nirenberg and Matthaei. A considerable amount of scientific study is currently being devoted to this *in vitro* protein synthesis system in an attempt to elucidate the structure and function of the ribosome with all of its attendant amino acid polymerization paraphernalia.

In the investigation here described, a technique used for *in situ* chemical modification of column-chromatographed *Escherichia coli* ribosomes is examined. This technique was developed in an attempt to clarify the structure of the bacterial ribosome. It was therefore necessary to work with pure ribosomes in a reproducible *in vitro* assay situation. Methods allowing: (1) isolation of chromatographically pure ribosomes;

TABLE 1. FACTORS AFFECTING RIBOSOMAL FUNCTION.

FACTOR	SOURCE	FUNCTION
F_3	Ribosome wash	Binding of mRNA
$F_1 - F_2$	Ribosome wash	Binding of fMet-tRNA
T_u, T_s — T	Cell supernatant	Formation of the GTP-tRNA-T complex; and transfer to ribosomes
Peptidyl Transferase	One of the 50S ribosomal proteins	Peptidyl transfer to aminoacyl-tRNA (formation of the peptide bond)
G and GTP	Cell supernatant	Translocation function; release of P_i from GTP
R_1 and R_2	Cell supernatant	Release of finished polypeptide from tRNA due to the stop codons

This table was taken from a prior published source (Lipmann, 1969). Specific references to all of the factors mentioned in the table are given in the text (with the exception of the release factors).

and (2) an efficient, reporducible assay of their biological activity were devised by the author. These techniques, and studies relating to these techniques, are presented in Appendix 1. Appendix 2 contains a potentially important discovery made during the course of the principal investigation which would be out of place elsewhere.

Before proceeding with a description of the chemical modification technique, and implications about ribosomal structure its application made possible, it is in order to review the salient features of cellular protein synthesis -- the so-called "central dogma".

The "central dogma"

It is clear that the majority of chemical information unique to a particular organism is carried in coded form by the cell as doubly-stranded deoxyribonucleic acid (DNA). Elucidation of the mechanism whereby the information contained in cellular DNA is decoded, resulting in the accurate synthesis of specific proteins, constitutes a major triumph of recent scientific endeavor. The fundamental outlines of the decoding mechanism are now assumed to be known.

Cellular DNA is not translated directly. Instead, a molecule of RNA is constructed which is complementary to one of the two DNA strands. It is this RNA strand, called messenger RNA (mRNA), that is translated into protein by the cellular apparatus responsible for protein synthesis. This apparatus consists of various adaptor molecules interacting with a "black

SECOND LETTER— FIRST LETTER	U	C	A	G	THIRD LETTER
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	ochre	umber	A
	Leu	Ser	amber	Try	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

Figure 1. Current three-letter codon assignments. The letters U, C, A, and G refer to the organic bases which make up RNA. Three letters (a first letter, a second letter, and a third letter) make up the code. For example, the codon AAA represents the amino acid Lys (lysine) in the figure. The Met (methionine) codon (AUG) specifies both methionine and N-formylmethionine (fMet). Ochre, amber, and umber refer to specific stop codons. These codons signal to the ribosome a set of stopping procedures (such as esterase splitting of the C-terminal amino acid from its tRNA, and ribosomal dissociation into subparticles).

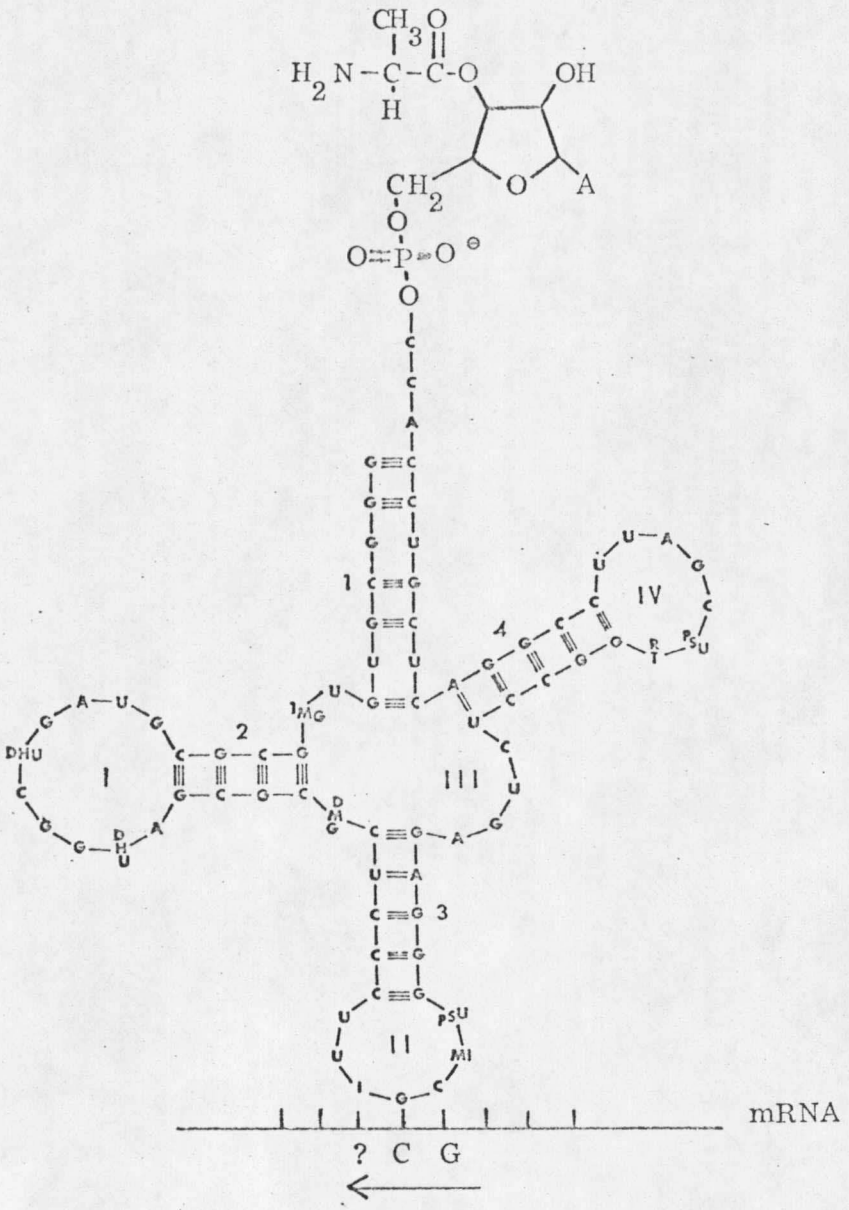

Figure 2. The nucleotide sequence of alanyl-tRNA (from Lipmann, 1969). This sequence was established by Holley and co-workers (1965). At the top of the figure, a sugar-phosphate backbone enlargement has been drawn in, illustrating the manner in which the amino acid is connected to the 3'-hydroxyl of the terminal ribose. At the bottom of the figure, the codon-anticodon interaction is shown.

The "black box"

In the electron microscope, the functional bacterial ribosomal particle appears spherical with a diameter of 200 - 220 Å (Huxley, 1960); this is the metabolically active 70S particle, so-called because it has a sedimentation coefficient of approximately 70 (Tissieres, 1959) -- measured by standard methods when ribosomal preparations are subject to a centrifugal force field in an analytical ultracentrifuge. The molecular weight of the 70S bacterial particle is approximately 2.3 million daltons.

It was early recognized that dialysis os the 70S particle against water resulted in the formation of 50S and 30S subparticles having an RNA to protein ratio identical to that of the 70S. This "dialyzable" factor has subsequently been identified as magnesium ion (Chao, 1957), and it is now known that the dissociation induced by low magnesium ion concentrations is completely reversible upon addition of sufficient magnesium salt (Hamilton, 1960).

The 50S subunit has been shown to contain two species of RNA -- the 23S (Tissieres, 1959), and the 5S (Rosset, 1964) -- and at least nineteen unique proteins (Osawa, 1969). The 30S subunit has been shown to contain one species of RNA -- the 16S (Tissieres, 1959) -- and twenty-one unique proteins (Hardy, 1969; Craven, 1969).

Ribosomal RNA

The best physical characterization of ribosomal RNA (rRNA)

has been done by Stanley and Bock (Stanley, 1965). They concluded that the 16S and 23S rRNA's were separate species formed of continuously-covalent phosphodiester linkages. From radius of gyration data they concluded that rRNA was coiled to a roughly spherical shape in solution; from sedimentation equilibrium measurements they ascertained that the molecular weight of 16S rRNA was 0.55×10^6, while that of 23S rRNA was 1.07×10^6. The rRNA species differ in their nucleotide composition and apparently have few, if any, sequences in common (Apirion, 1967). McIlreavy and Midgley have estimated the chain length of the 16S and 23S rRNA's, and on the basis of chemical data, found them to be identical (McIlreavy, 1967). Sykes has offered some evidence that the discrepancy between molecular weight and chain length may be due to the fact that the 23S rRNA is a dimer (Sykes, 1965), but resolution of this point awaits the chemical sequencing of the rRNA's. The complete sequence of 5S rRNA (molecular weight 35,000) has been determined by Brownlee (Brownlee, 1967), but its function remains obscure. It should be noted that Midgley and McIlreavy have reported the base content of rRNA to be medium dependent (McIlreavy, 1967).

Ribosomal protein

Traut (1967), and Fogel and Sypherd (Fogel, 1968) separated the ribosomal protein associated with the 30S subunit, finding 18 - 21 apparently unique species. Otaka (1968), Itoh (1968), and Osawa (1969) have resolved the 50S subparticle

proteins into nineteen components using disc electrophoresis and carboxymethylcellulose column chromatography.

A significant extension of this early work has been made by Traub and Nomura (Traub, 1968), who have reconstructed a functionally active 30S subparticle from 16S rRNA and 30S proteins. Their work may be represented schematically as:

Dissociation

$$30S \xrightarrow[\text{gradient}]{\text{cesium chloride}} \text{split protein (SPr)} + \text{core particle (CP)}$$

$$CP \xrightarrow[\text{extraction}]{\text{phenol}} 16S\ rRNA$$

$$CP \xrightarrow[4\ \underline{M}\ LiCl]{8\ \underline{M}\ \text{urea}} \text{core protein (CPr)}$$

Reconstitution

$$16S\ rRNA + CPr \xrightarrow[37^\circ\ C.]{\text{incubation}} CP \xrightarrow{SPr} 30S$$

Reversing the order of protein addition did not cause any decrease in activity, i.e., the 16S rRNA and 30S proteins together constitute a self-organizing unit. This interpretation has recently been confirmed from further studies by Nomura (Traub, 1968), on the thermodynamics and kinetics of the 30S particle reconstitution. "Hybrid" 30S particles may also be reconstituted using rRNA from one source and 30S proteins from another (Nomura, 1968).

Traub and Nomura have shown that 50S proteins (prepared by methods identical to those used in preparing 30S proteins)

do not substitute for 30S proteins, and that 23S rRNA does not substitute for 16S rRNA. Reconstitution work has yet to be done for the 50S ribosomal subunit.

Factors affecting ribosomal function

The synthesis of a natural protein is thought to take place according to the following sequence: (1) mRNA is bound to a 30S ribosomal subunit (Brenner, 1961), and the initiating codon AUG calls for the peptide initiator formyl-methionyl-tRNA (Leder, 1966). The 50S subunit subsequently combines with the 30S-mRNA complex (see Figure 3). The initiation factors F_1, F_2, and F_3 (see Table I) are operative in this phase, but their exact mode of action is not clear. It is known, however, that they are synergistic for natural mRNA and formyl-methionyl-tRNA (fMet-tRNA) binding (Stanley, 1966), but are not needed for poly U or polyadenylic (poly A) attachment to ribosomes (Nakamoto, 1966). (2) The arrangement depicted in Figure 3 now allows the proper aminoacyl-tRNA (Phe-tRNA in this case) to bind to the "open" codon (UUU in Figure 4a). This binding is catalyzed by factor T and GTP (see Table I), apparently through the formation of an aminoacyl-tRNA-GTP-T complex (Ravel, 1967).

The situation shown in Figure 4a is thought to be transitory, however, and peptide bond formation (peptidyl transfer) immediately takes place (see Figure 4b), apparently catalyzed by a ribosomal transferase factor (Traut, 1964). (3) Translocation now occurs during which the growing peptide of Figure