



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  
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Abstract:

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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 thiocyanines was shown to be a dark inactivation of their biological activity.

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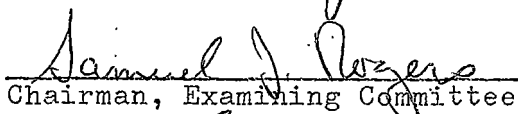
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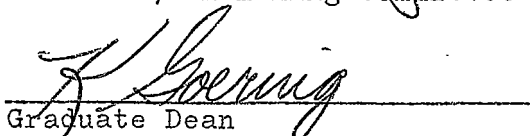
in

Chemistry

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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 involvement 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

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.

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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
$\begin{array}{c} T_u \\   \\ T_s \end{array} - T$	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 (forma- tion 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 poly- peptide 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, reproducible 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

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

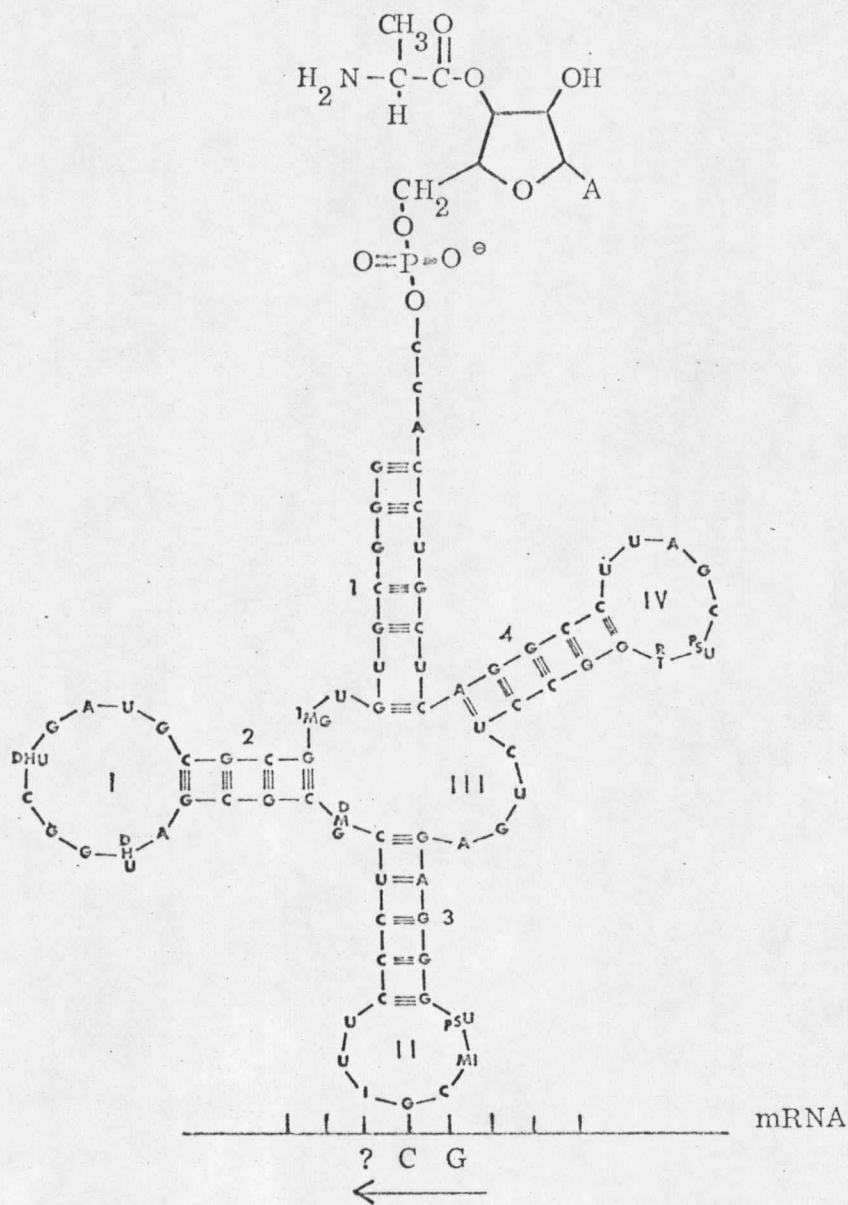


Figure 2. The nucleotide sequence of alanyl-tRNA (from Lipmann, 1969). This sequence was established by Holley and co-workers (1965). At the top of the figure, a sugar-phosphate backbone enlargement has been drawn in, illustrating the manner in which the amino acid is connected to the 3'-hydroxyl of 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 of 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  $0.55 \times 10^6$ , while that of 23S rRNA was  $1.07 \times 10^6$ . 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.



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

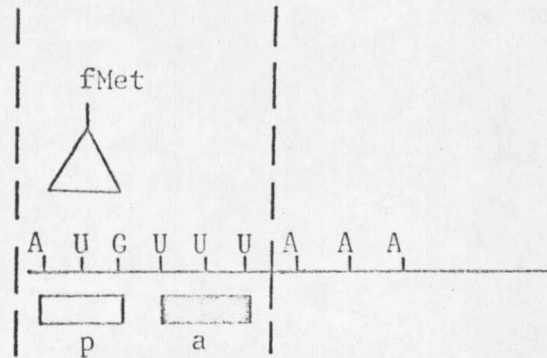


Figure 3. Protein synthesis initiation on the ribosome. In this diagrammatic representation, the 'p' and 'a' sites (the peptidyl tRNA site and the aminoacyl-tRNA site, respectively) shown as blocks represent hypothetical decoding and bond-forming locations. Evidence for the existence of these two sites came from tRNA-binding studies. The 30S subunit itself (in the presence of poly U) bound phenylalanyl-tRNA, in the ratio one tRNA per 30S particle, while the binding of Phe-tRNA to the 50S particle was negligible (Kaji, 1966). However, addition of 50S subunits to a 30S preparation resulted in a two-fold stimulation of the Phe-tRNA binding capacity of the 30S subunit (Suzuka, 1966). Work using EDTA-induced ribosomal dissociation was interpreted to demonstrate that there is only one tRNA binding site on the 30S (the decoding site, or 'a' site), therefore leaving the second tRNA binding site to the 50S (Suzuka, 1967). This latter site was the condensing site (peptide bond-forming site), or 'p' site shown above.

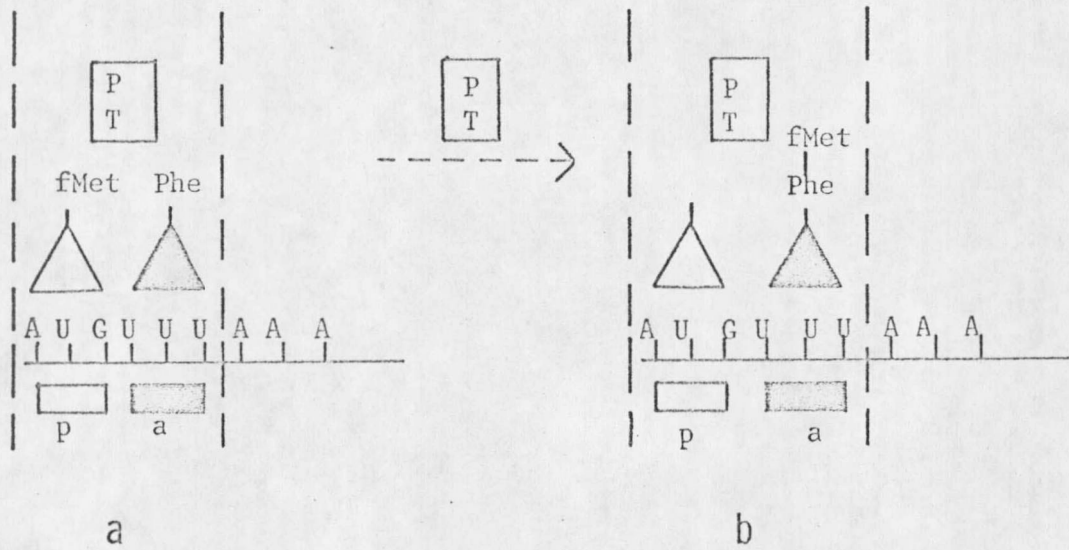


Figure 4. The bond-forming sequence on the ribosome. In this diagrammatic representation, all of the symbols used are the same as those used in Figure 3, with the exception that  $\begin{matrix} \text{P} \\ \text{T} \end{matrix}$  represents the peptidyl transferase enzyme located on the 50S particle.

4b is moved (translocated) to the 'p' site (see Figure 5). Lipmann and others (Nishizuka, 1966; Nathans, 1962) have argued that the energy required for this translocation is provided by hydrolysis of GTP, somehow mediated by factor G. (see Table I). The translocase step restores the original complex shown in Figure 3, and permits the next codon (AAA in this case) to move into the 'a' site, thus becoming "open" and allowing sequential translation of the message. This model is purposely vague as to structural details because these details are currently unknown.

#### The investigation rationale

Of all the functions assigned to the ribosome, the only one associated with a ribosomal constituent is the peptidyl transferase, shown by Monro (Monro, 1968) to be one of the 50S proteins. It was to extend this detailed structure / function relationship that the current investigation was undertaken.

Inherent in the model of ribosomal action presented above is the notion that the ribosome is operationally an active surface. Overturn of the "inert scaffold" picture of the ribosome prevalent a few years ago was made possible by the assignment of the peptidyl transferase function to a 50S protein (the particular protein, or proteins -- which of the nineteen possible -- is unknown).

The difficulty facing this active surface approach is that no specific ribosomal constituent has been assigned to any of the several necessary functions performed by the ribosome. The

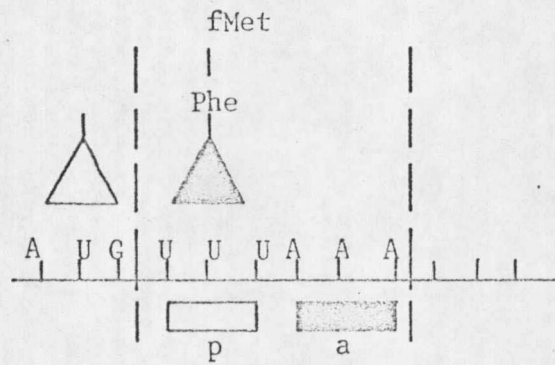


Figure 5. The translocase sequence on the ribosome. This figure is a diagrammatic representation of ribosomal protein synthesis immediately following the translocase operation performed by the ribosome during protein synthesis. All of the symbols used are the same as for Figure 3.

quandary of researchers in this area is this: The ten or more functions of the ribosomal conglomerate can be understood only as part of the whole, integrated, active ribosomal unit. Upon dissociation of this functional ribosome into discrete protein and rRNA species, all functions are lost, and therefore no structure / function assignments can be made since no individual assays exist.

There are only two examples which indicate that a ribosomal alteration can be correlated in an obvious manner to a specific ribosomal protein. Traub and Nomura (Traub, 1968) have shown that the alteration induced by the streptomycin resistance mutation ( $Sm^R$ ) resides in one of the 30S core proteins -- protein P10 -- and not in the rRNA or in the split proteins (SPr) of the particle (Traub, 1966). It is anticipated by these authors that protein P10 will be important to initiation, since reconstituted ribosomal particles lacking P10 were inactive with natural message, but showed some activity with synthetic message.

The second example concerns the "K-character" protein reported by Flaks (Flaks, 1966). One of the 30S ribosomal proteins -- protein P8 -- of E. coli strain B has a greater electrophoretic mobility than the homologous protein in E. coli strain K. Recently Birge reported that the genetic locus which determines the electrophoretic mobility of protein P8 was the structural gene for this protein (Birge, 1969). The function of protein P8, however, remains obscure.

Ideally, what was needed for an extension of the active surface approach was the development of a specific reagent which clearly inactivated a particular ribosomal function in some irreversible manner -- presumably by modification of the chemical groups which make up the catalytic surface. The constituent moiety shown necessary for a particular function and affected by this chemical agent would thus be visible using conventional chemical and physical techniques when applied to the proteins and rRNA of dissociated ribosomes.

The technique of dye-sensitized photo-inactivation of the ribosome developed in this investigation in large measure meets these specific reagent requirements. This technique was the method of choice because the interaction of radiation with molecules can lead to very specific chemical reactions. High-impact, large energy-transfer processes, however, such as irradiation of molecules with X-rays or "hard" ultraviolet radiation normally lead to a wide variety of products because the radiation has enough energy to rupture all types of chemical bonds.

It is possible nevertheless to introduce into a system radiant energy of 30 - 100 kilocalories per mole through the use of sensitizers. Very generally, energy is absorbed by the sensitizer and transformed by a variety of degradative steps to a long-lived, potentially reactive intermediate. Under favorable conditions, this intermediate may react chemically with a particular substrate molecule, and since the energy of

the reactive intermediate is low, the chemical reaction(s) initiated by the sensitizer can be very specific.

This technique of applying a sensitizer-induced chemical reaction to inactivate an enzymatic function has been called "photodynamic action" (Tappeiner, 1904).

## MATERIALS AND METHODS

### Buffer recipes

Buffer B: 0.05 M potassium chloride (KCl); 0.011 M magnesium acetate ( $\text{Mg}(\text{OAc})_2$ ); 0.10 M Tris(hydroxymethyl)aminomethane (Tris); pH adjusted to 7.8 with glass-distilled (g. d.) hydrochloric acid (HCl).

Buffer N: 0.08 M ammonium chloride ( $\text{NH}_4\text{Cl}$ ); 0.011 M  $\text{Mg}(\text{OAc})_2$ ; 0.10 M Tris; pH adjusted to 7.8 with g. d. HCl.

Buffer OB: 0.50 M  $\text{NH}_4\text{Cl}$ ; 0.002 M  $\text{Mg}(\text{OAc})_2$ ; 0.01 M Tris; pH adjusted to 7.8 with g. d. HCl.

Buffer OC: 0.25 M  $\text{NH}_4\text{Cl}$ ; 0.005 M  $\text{Mg}(\text{OAc})_2$ ; 0.01 M Tris; pH adjusted to 7.8 with g. d. HCl.

Buffer OD: 0.50 M  $\text{NH}_4\text{Cl}$ ; 0.01 M  $\text{Mg}(\text{OAc})_2$ ; 0.01 M Tris; pH adjusted to 7.8 with g. d. HCl.

Buffer pH 5K: 0.05 M KCl; 0.011 M  $\text{Mg}(\text{OAc})_2$ ; 0.10 M Tris; pH adjusted to 5.2 with g. d. acetic acid (HOAc).

Buffer pH 5N: 0.05 M  $\text{NH}_4\text{Cl}$ ; 0.011 M  $\text{Mg}(\text{OAc})_2$ ; 0.10 M Tris; pH adjusted to 5.2 with g. d. HOAc.

Buffer pH 5'K: 0.05 M KCl; 0.011 M  $\text{Mg}(\text{OAc})_2$ ; 0.01 M Tris; pH adjusted to 5.2 with g. d. HOAc.

Buffer pH 5'N: 0.05 M  $\text{NH}_4\text{Cl}$ ; 0.011 M  $\text{Mg}(\text{OAc})_2$ ; 0.01 M Tris; pH adjusted to 5.2 with g. d. HOAc.

Pestka and Nirenberg subunit buffer (Pestka, 1966a): 0.0002 M  $\text{Mg}(\text{OAc})_2$ ; 0.01 M Tris; pH adjusted to 7.8 with g. d. HCl.

Buffer NSP: 0.0004 M spermidine; 0.02 M putrescine; 0.08 M

$\text{NH}_4\text{Cl}$ ; 0.011 M  $\text{Mg}(\text{OAc})_2$ ; 0.10 M Tris; pH adjusted to 7.8 with g. d. HCl.

Buffer S: 0.10 M Tris; 0.01 M  $\text{Mg}(\text{OAc})_2$ ; pH adjusted to 7.8 with g. d. HCl.

Buffer T: 0.01 M Tris; 0.001 M  $\text{Mg}(\text{OAc})_2$ ; pH adjusted to 7.8 with g. d. HCl.

Buffer TAK: 0.01 M Tris; 0.001 M  $\text{Mg}(\text{OAc})_2$ ; pH adjusted to 7.8 with g. d. HCl.

Takanami low-magnesium buffer (Takanami, 1967): 0.01 M Tris; 0.00002 M  $\text{Mg}(\text{OAc})_2$ ; pH adjusted to 7.8 with g. d. HCl.

The presence of a plus sign (+) when used in designating a buffer (for example, NSP+) signified that the buffer had been made 0.006 M in 2-mercaptoethanol. A minus sign (-) or the letter designation alone (for example, N or N-) meant that the buffer was 2-mercaptoethanol-free.

#### Buffer preparation

To insure that buffer solutions used throughout this investigation were uniform, the following procedure was used when preparing all buffers:

(1) Dry reagents were weighed out and added to a clean beaker.

(2) Approximately 800 ml. of g. d. water was added to the dry reagents. Any wet reagents (with the exception of pH-adjusting acid and 2-mercaptoethanol) were added at this time.

(3) The contents of the beaker were dissolved, the solution transferred to a one-liter volumetric flask, and the buffer volume adjusted to 1000 ml. with additional g. d. water.

(4) The contents of the volumetric flask were poured back into the original beaker, and pH adjustments were made.

(5) Two drops of solution bentonite (see below) were added per liter of buffer and stirred into suspension.

(6) After the solution bentonite had worked in the buffer for at least two hours, the buffer was filtered through a Millipore filtration apparatus equipped with a 50 mm. Millipore filter (0.45  $\mu$  pore size), and collected in an appropriate bottle. The Millipore apparatus, filter, and bottle had been made sterile and ribonuclease-free by prior autoclaving.

(7) Appropriate amounts of 2-mercaptoethanol were added, if needed, to complete the buffer preparation.

#### Preparation of solution bentonite

Bentonite (Wilkinite), a colloidal native hydrated aluminum silicate clay, principally  $\text{Al}_2\text{O}_3 \cdot 4\text{SiO}_2 \cdot \text{H}_2\text{O}$ , has been shown to be effective in removing the activity of ribonuclease enzymes from natural preparations (Fraenkel-Conrat, 1961).

Solution bentonite was used in this study for the preparation of ribonuclease-free buffers, and for soaking apparatus to be used in the preparation of ribosomes and other RNA-containing materials.

Two grams of bentonite (purchased as a crude powder from Fisher Scientific Company) was suspended in 40 ml. of g. d.

water, then spun for 15 minutes at 2,500 r.p.m. (800 x g) in the SS-1 rotor of the Servall Superspeed Centrifuge. The supernatant from this step was centrifuged for 20 minutes at 8,500 r.p.m. (9,200 x g) in the SS-1 rotor. The sediment was resuspended in the buffer of choice for 48 hours at room temperature.

This resuspended bentonite was spun for 15 minutes at 2,500 r.p.m., and the resulting supernatant for 20 minutes at 8,500 r.p.m. (both in the SS-1 rotor). The sediment from the last step was again resuspended by standing in buffer for 48 hours at room temperature.

Finally, the resuspended bentonite was spun for 30 minutes at 8,500 r.p.m. in the SS-1 rotor and the supernatant discarded; the sediment was suspended in g. d. water or buffer, and bottled. This suspension contained no discernible precipitate after six months or more storage in a stoppered dropping bottle.

#### Removal of ribonuclease

Ribonuclease (RNAase) was removed from glass or metal ware by: (1) heating in a dry oven at 240° C. for four hours (Chao, 1961); or (2) autoclaving for 30 minutes (Brown, 1962). RNAase was removed from plastic ware by: (1) autoclaving for 30 minutes (if the plastic could resist such treatment); or (2) treatment for 15 minutes with 15% hydrogen peroxide (Clark, 1967); or (3) soaking for several hours in bentonite solution.

### Dyes

All dyes used were obtained from Fisher Scientific Company except the following: acridine orange (Allied Chemical); neutral red (Eastman); chlorophyllin (Na-K-Cu) chlorophyll (Nutritional Biochemicals Corporation); malachite green (Schaar and Company); crystal violet (Matheson, Coleman and Bell); FMN (Calbiochem). Thiopyronine was a gift from Dr. J. S. Bellin, Polytechnic Institute of Brooklyn, and the thiocyanines were a gift from Dr. A. C. Craig, Montana State University. The dyes were used without further purification.

### Glass-distilled hydrochloric acid

Seven hundred and fifty milliliters of reagent-grade hydrochloric acid (DuPont) were diluted with an equal amount of g. d. water, this mixture placed in a three-neck flask, and distilled. The first 500 ml. of distillate were saved for general laboratory use; the second 500 ml. was the fraction used for making buffers throughout this investigation; and the last 500 ml. remaining in the three-neck flask were discarded.

### Glass-distilled water

House-distilled water was re-distilled in a Corning Model AG-2 Water Distillation Apparatus and the distillate collected in Pyrex carboys. This water was used for the preparation of all buffers used in this investigation.

### Preparation of ribosomes

All reagents, buffers, and glass, plastic, or metal ware used in this preparation were made RNAase-free by appropriate

treatment.

One hundred and sixty to two hundred grams of frozen Escherichia coli cells (strain B or Q13, purchased from General Biochemicals, Incorporated) were lysed according to either cell-lysing procedure (see below). The cell lysate was placed in centrifuge tubes appropriate for use in the Type 30 rotor of the Beckman Model L-2 Ultracentrifuge, and spun for 2 hours at 18,000 r.p.m. (30,000 x g) to remove fine cell debris. The top 90% of the supernatant was removed with a syringe, transferred to tubes suitable for the Type 50-Ti rotor of the Model L-2, and spun for 3 hours at 50,000 r.p.m. (170,000 x g). The top 80% of the supernatant obtained in this pelleting operation was collected and saved as crude S-100.

Two milliliters of buffer OB were added to each centrifuge tube containing a ribosomal pellet, and this pellet was allowed to dissolve by standing 12 - 14 hours at 4° C.

The crude S-100 fraction was immediately centrifuged for 3 hours at 50,000 r.p.m. in the Type 50-Ti rotor, and the top two-thirds of the supernatant, the S-100 fraction, was removed, pooled, bottled in 4 - 5 ml. aliquots, and stored at -90° C. until used to make the pH 5 fraction (see below).

The dissolved ribosomal pellet was spun for 15 minutes at 25,000 r.p.m. (42,000 x g) in the Type 50-Ti rotor to remove any heavy material. Following this low-speed wash, the supernatant was poured into fresh centrifuge tubes and spun for 4 hours at 50,000 r.p.m. in the Type 50-Ti rotor. Subsequent to

this high-speed pelleting, the top 80% of the supernatant was discarded, and 2 ml. of buffer OC were added to each tube containing a pellet. The pellet was dissolved by standing 2 - 6 hours at 4° C. with occasional stirring.

Occasionally the ribosomal pellet at this stage contained dark-colored material. In this case, an additional 2 ml. of buffer OB were added to each tube, the ribosomes allowed to dissolve over an 8 - 10 hour period, and an additional low-speed wash, followed by a high-speed pelleting, was carried out before the final addition of buffer OC.

The dissolved ribosomes were combined, their volume adjusted to approximately 50 ml. by the addition of buffer OC, and optical density readings were taken to determine the concentration of the ribosomal solution, based on 16.6 optical density units per mg. of ribosomal material. Two hundred milligrams of ribosomes (in solution) were introduced to the top of a diethylaminoethyl (DEAE)-cellulose column prepared previously (see below). The column was washed with 500 ml. of buffer OC, and the ribosomes eluted with 500 ml. of buffer OD. During washing and elution, aliquots of 15 - 20 ml. were collected using an automatic collecting device. The ribosomal fraction was opalescent.

The collected chromatographed ribosomes were pelleted by centrifugation for four hours at 50,000 r.p.m. in the Type 50-Ti rotor. The pelleted ribosomes were taken up in the buffer of choice (usually N-) to the desired concentration (10 - 30

mg./ml.) using optical density criteria, and stored at  $-90^{\circ}$  C. in appropriate aliquots (0.25 - 1.0 ml.)

A protocol for this ribosome preparation is presented in Appendix 1.

#### Cell lysing procedures

Procedure A. One hundred and sixty to two hundred grams of frozen E. coli cells were mixed with enough cold RNAase-free buffer N+ to bring the total volume of cells plus buffer to a maximum of 340 ml. Deoxyribonuclease I (0.6 mg., obtained from Worthington Biochemical Corporation) was added to this mixture, and the mixture was allowed to stand overnight at  $4^{\circ}$  C. In the morning, the mixture was passed through a chilled French pressure cell (American Instrument Company) equipped with a one-inch piston, at a pressure of 9,000 p.s.i., and the cell lysate was collected in a chilled beaker. Cell debris was removed by centrifugation for 45 minutes at 18,000 r.p.m. in the Type 30 rotor of the Beckman L-2 centrifuge, the lysate poured off and pooled.

Procedure B. E. coli cells were added to a large mortar in approximately 80 g. quantities, covered with a thin layer of abrasive-grain levigated alumina (Norton Company), and crushed with a pestle while still frozen until the mass of cells was slightly thawed. Just enough alumina was added as needed to keep the cells damp, working continuously with the pestle. This operation was continued until the cells had a fine, granular texture, and were very nearly thawed completely. Fifty

milliliters of buffer N+ were added at this time, and quickly mixed in with the cells and alumina to form a thick paste. Grinding was resumed and continued until the paste was homogeneous. The paste was poured from the mortar into a beaker, and the mortar charged with an additional 80 g. portion of cells. One hundred and sixty to two hundred grams of cells were ground in this manner, using about 350 g. of alumina. The mortar was rinsed at the end of the grinding with two 25-ml. portions of buffer N+.

The combined alumina-cell paste was centrifuged at 18,000 r.p.m. for 45 minutes in the Type 30 rotor to bring down the alumina and coarse cell debris. The crude lysate was poured off the pelleted alumina, and 0.6 mg. of deoxyribonuclease was added to it. The deoxyribonuclease was allowed to work for 90 minutes before the lysate was subjected to further treatment.

#### Column preparation for DEAE-cellulose

DEAE-cellulose, 0.69 meq./g., was treated with 0.1 M sodium hydroxide solution, then washed with g. d. water to neutrality employing a Buchner funnel. The material in the funnel was washed with 95% ethanol (three times), then with g. d. water (three times). Following a second wash with sodium hydroxide solution, and an extensive water rinse, the DEAE-cellulose was autoclaved for 15 minutes as a slurry with buffer OC.

The autoclaved DEAE-cellulose slurry was packed into a glass column of 50 x 2.5 cm. under air pressure of 5 - 10 p.s.i.

at 4° C. Glass-fiber paper was placed above the packed column to prevent disturbance of the bed.

Preparation of ribosomal subunits

Takanami procedure (Takanami, 1967). The ribosome-containing eluent from DEAE-cellulose chromatography (see ribosome preparation above) was collected, transferred to centrifuge tubes suitable for the Beckman Type 50-Ti rotor, and spun for 6 hours at 40,000 r.p.m. (100,000 x g) in the Model L-2 centrifuge. The upper four-fifths of the supernatant was carefully removed and discarded, and the remaining fluid was gently shaken for a few seconds to resuspend the upper layer of the pellet. This resuspended fraction contained mainly the 30S subunit, while the remaining pellet contained principally the 50S material. The 30S fraction was poured into fresh tubes, diluted with buffer TAK+, and centrifuged at 40,000 r.p.m. for 5 hours in the Type 50-Ti rotor. Following centrifugation, the upper four-fifths of the supernatant was again removed and discarded, and the pellet gently shaken in the remaining fluid. The dissolved material was decanted and bottled as the crude 30S fraction.

Separately, the remaining pellet from the first centrifugation (the 50S fraction) was resuspended in buffer TAK+ and centrifuged at 40,000 r.p.m. for 2 hours in the Type 50-Ti rotor. Following centrifugation, the supernatant was decanted, most of the pellet resuspended in a small amount of buffer TAK+, and the dissolved material was bottled as crude 50S

fraction.

Each crude fraction still contained about 10% of the complementary particle (as determined by analysis in the Beckman Model E analytical ultracentrifuge). Further purification was achieved by sucrose density gradient centrifugation, using 5 - 20% linear gradients prepared in buffer TAK+ by means of a gradient former. Approximately 1.5 - 2.0 ml. of crude fraction were layered on top of 16-ml. gradients which were formed in tubes suitable for the SW 25.3 rotor, and subjected to centrifugation in the Beckman Model L-2 at 25,000 r.p.m. (70,000 x g) for 5 - 6 hours. Subsequently, the centrifuge tubes containing the separated material were punctured by a density gradient fractionator which allowed drop-wise collection of the gradient. According to the profiles obtained upon ultraviolet analysis (at 260 m $\mu$ ) of the collected aliquots, the fractions corresponding to each subunit were pooled, concentrated by centrifugation at 50,000 r.p.m. in the Type 50-Ti rotor, diluted to the desired concentration with buffer N-, made up in appropriate aliquots, and stored in a freezer at -90° C.

Sepharose 4B procedure. Sepharose 4B (Pharmacia Fine Chemicals) was allowed to equilibrate with buffer TAK+ for 24 hours, then packed into a 50 x 1.5 cm. cylindrical glass column. Approximately 6 mg. of chromatographed ribosomes (in buffer TAK+) were layered on the top of the packed column, and eluted with buffer TAK+. On the basis of the ultraviolet profile obtained from the eluent, a center-cut of the fraction

corresponding to each subunit was collected, several runs were pooled, and the pooled fractions concentrated by centrifugation at 50,000 r.p.m. for 4 hours in the Model 50-Ti rotor of the Beckman Model L-2 Ultracentrifuge. These concentrated center-cuts were diluted to the desired concentration with buffer N-, and stored in appropriate aliquots in a freezer at -90° C.

#### Dialysis of ribosomes

Occasionally a ribosomal preparation would contain a high proportion of 70S particles. These preparations were dialyzed against a buffer which contained a low magnesium concentration before being used for the preparation of subunits. Appropriate dialysis buffers included: Pestka and Nirenberg subunit buffer; buffer T; and Takanami low-magnesium buffer. The dialysis was carried out for 4 - 6 hours, and the resulting dialysate was used as the starting material for subunit preparation.

#### Preparation of the pH 5 fraction

The pH 5 enzyme fraction was prepared by a modification of a method used previously (Julian, 1965a). All operations were carried out at 4° C.

A one-centimeter (diameter) glass chromatographic column equipped with an extra coarse supporting disc was loaded with a slurry (previously equilibrated with buffer pH 5N or pH 5K) of Sephadex G50 coarse beads (Pharmacia Fine Chemicals) to a packed-column height of 11 - 12 cm. Three to five milliliters of S-100 fraction (see ribosome preparation above) were passed into the column and displaced with buffer pH 5N or pH 5K. The

fraction of the eluent which contained a milky precipitate (usually about 5 ml. in volume) was collected in a 12-ml. conical centrifuge tube, 5 ml. of fresh buffer pH 5N or pH 5K were added to this tube, and the precipitate spun down in a clinical centrifuge.

The precipitate obtained was washed twice with buffer pH 5'N or pH 5'K by resuspension and recentrifugation, and was subsequently dissolved in 1.2 - 1.5 ml. of buffer N- or B-. The solution obtained by this procedure routinely contained a small amount of insoluble material, and the solution was therefore centrifuged continuously at mid-range speed in a clinical centrifuge until use. The bottom 0.2 ml. of this pH 5 preparation was not added to the incorporation ingredients.

#### Incorporation procedure

The incorporation of amino acids into peptides was carried out by incubating E. coli ribosomes in 12-ml. conical centrifuge tubes in the presence of C<sup>14</sup>-amino acid, a message appropriate to the amino acid, and the requisite energy sources. Because of the nature of this investigation, the incorporation ingredients were divided into three fractions.

Fraction 1. Buffer, ribosomes, and dye were initially added to a 12-ml. conical centrifuge tube immersed in ice. Chemical modification was normally attempted on this fraction only.

Fraction 2. This fraction contained the energy sources, tRNA, pH 5 enzymes, and C<sup>14</sup>-amino acid. It was kept in a large

vessel immersed in ice.

Fraction 3. This fraction consisted of message only, and was kept in a dispenser apparatus designed to deliver a 3  $\mu$ l. drop.

Normal procedure. Following the desired manipulation of Fraction 1, an appropriate amount of Fraction 2 was added in the dark and stirred into solution. Initiation of the incorporation assay was begun by adding Fraction 3 at timed intervals. The final volume of the assay was one milliliter, and this incorporation mixture was normally incubated at 37° C. for 30 - 40 minutes.

Message-present procedure. When it was necessary to have the message present during photooxidation, initiation of peptide synthesis was accomplished by adding Fraction 2 to the combined Fractions 1 and 2.

Fraction recipes

Fraction 1. Three hundred and thirty microliters of buffer (either B- or N-); 100  $\mu$ l. of standard dye solution (which contained 0.2  $\mu$ moles of dye); and 20  $\mu$ l. of ribosomes (approximately 500  $\mu$ g.) made up this fraction.

Fraction 2. Fifty microliters each of ATP (P-L Biochemicals Incorporated); GTP (Sigma Chemical Company) and CTP (P-L Biochemicals Incorporated); PEP (Calbiochem); pyruvate kinase (Calbiochem); and C<sup>14</sup>-amino acid (Amersham/Searle; Miles Laboratories) standard solutions plus 100  $\mu$ l. each of sRNA (Schwarz Bioresearch, Incorporated); pH 5 fraction (see above);

and either B or N buffer containing 0.06 M 2-mercaptoethanol standard solutions constituted this fraction.

Fraction 3. Three microliters of a standard solution of mRNA.

Standard solution recipes

ATP. 62.3 mg./ 10 ml. buffer (as the sodium salt).

GTP and CTP. 18.09 mg. GTP and 17.97 mg. CTP/ 100 ml. buffer (as the sodium salts).

PEP. 15.75 mg./ 10 ml. buffer (as the sodium salt).

Pyruvate kinase. 300 E. U./ 100 ml. buffer.

sRNA. 4 mg./ml. buffer.

Poly A. 15.5 mg./ml. distilled water.

Poly U. 8 mg./ml. distilled water.

Polylysine. 2 mg./ml. distilled water.

Using these standard solutions in the amounts indicated resulted in each incorporation trial tube containing the following ingredients in one milliliter: 0.5  $\mu$ moles ATP; 0.015  $\mu$ moles GTP and CTP; 0.25  $\mu$ moles PEP; 0.15 E. U. (one microgram) of pyruvate kinase; 0.000415  $\mu$ moles of C<sup>14</sup>-amino acid; 100  $\mu$ l. of pH 5 enzyme solution; 0.6 mg. of E. coli sRNA; approximately 500  $\mu$ g. of E. coli ribosomes; 6  $\mu$ moles of 2-mercaptoethanol; 0.2  $\mu$ moles of dye; and either 50  $\mu$ g. of poly A (Miles Laboratories) or 25  $\mu$ g. of poly U (Miles Laboratories).

Photooxidation procedure

Photooxidation of E. coli ribosomes contained in a small volume of buffer with the required concentration of dye (see

text for specific quantities in each instance) was carried out at 0° C. in 12-ml. conical centrifuge tubes placed in an ice bath positioned approximately 10 cm. directly in front of the lens of a 500-watt slide projector (Viewlex projector equipped with an f3.5 Luxtar-K lens; Viewlex Corporation). The projector was operated for the times indicated in the text, after which the tubes were placed in light-free aluminum envelopes equipped with removable caps. The remainder of the incorporation ingredients was added, and peptide synthesis initiated by the addition of either Fraction 2 or Fraction 3.

#### Plating and counting procedures

Lysine peptides. Incorporations using poly A-directed lysine polymerization were terminated by the addition of 0.04 mg. of polylysine, 6 ml. of tungstate solution (0.3 N trichloroacetic acid (TCA); 0.01 N sodium tungstate), and 0.2 ml. of 10% TCA solution, in the order listed.

After thirty minutes, tubes containing the precipitated material were centrifuged for 5 minutes at top speed in a clinical centrifuge, and the supernatant drawn off the pelleted material by means of a Pasteur pipette attached to an aspirator. Six milliliters of fresh tungstate solution was added, the precipitate dispersed with a glass stirring-rod, and the resulting suspension heated for 15 minutes at 85 - 90° C. The tubes were then cooled, 0.1 ml. of 10% TCA solution was added to each tube, and the tubes allowed to stand for at least one hour in the cold.

The resulting precipitate was centrifuged for 5 minutes at top speed in a clinical centrifuge, the supernatant drawn off as before, and the pellet dissolved in one milliliter of 0.2 N potassium hydroxide solution. Six milliliters of tungstate solution were added to each tube followed by the addition of one drop of concentrated hydrochloric acid. After standing in the cold for at least 30 minutes, the suspensions were filtered on Millipore filters (23.5 mm. in diameter, made by a hand-press from 0.45  $\mu$  pore size Millipore material), mounted in Pyrex Microanalysis Filter Holders (Millipore Corporation), washed seven times with 3 ml. of tungstate solution, removed from the filter holders and placed in appropriate ring-and-disc sets (RD-1, Tracerlab), dried for about one hour at approximately 80° C., and counted in a thin-window gas-flow counter (Model 6115 low-background automatic sample-changing planchette counter equipped with a decade scaler (Model 8703) and a printing lister (Model 8437); Nuclear-Chicago Corporation).

Phenylalanine peptides. Tubes containing a poly U-directed phenylalanine incorporation were terminated by the addition of one milliliter 10% TCA solution, and 5 ml. of 5% TCA solution added in the order listed.

Tubes containing the precipitated peptides were heated immediately for 15 minutes in a boiling water bath, cooled for one hour, centrifuged for 5 minutes at top speed in a clinical centrifuge, the supernatant drawn off the pelleted material by

means of a Pasteur pipette attached to an aspirator, and the pellet dissolved in 1.0 ml. of 0.2 N potassium hydroxide. Six milliliters of 5% TCA solution were added to each tube, and the tubes were allowed to stand in the cold for at least 30 minutes. The resulting suspensions were filtered, dried, and counted as for the lysine peptides above.

Messenger RNA binding procedure

Each trial tube contained the following ingredients: three hundred and forty microliters of buffer B-; 1.2 mg. of ribosomes (in 50  $\mu$ l. of solution); and 100  $\mu$ l. of Rose Bengal at the normal concentration (0.2  $\mu$ moles of dye). Following photooxidation (carried out in the normal fashion), 0.01  $\mu$ Ci. of  $C^{14}$ -poly U was added to each tube, and the mixture allowed to incubate either at 0° C. or 37° C. for 10 minutes.

Analysis of binding. Four hundred microliters of the incubation mixture presented above was layered on the top of a 5 - 20% sucrose density gradient. The gradient was prepared by running 16 ml. of a 5 - 20% linear sucrose gradient into a cellulose nitrate tube. After centrifuging for 20 hours at 25,000 r.p.m. in the Beckman SW 25.3 rotor of the Model L-2 centrifuge, the gradients were separated into 16-drop fractions directly into scintillation bottles. A 100  $\mu$ l. sample of each homogeneous 16-drop fraction was removed and pipetted into one milliliter of g. d. water contained in a numbered test tube. The remainder of the 16-drop fraction was dissolved in 15 ml. of toluene-based scintillation fluid (1100 ml.

toluene; 900 ml. ethanol; 168 ml. formamide; 6.4 grams 2,5-diphenyloxazole), and counted in a Beckman LS-100 ambient temperature scintillation spectrophotometer. The optical density of the contents of each numbered test tube was determined by monitoring the absorbance of each sample at 260 m $\mu$  in a Gilford multiple-sample absorbance recorder equipped with a Beckman DU-R monochrometer.

Transfer RNA binding procedure

The procedure used was based on one reported earlier (Pestka, 1968). Each trial tube contained the following ingredients: fifty microliters of GTP standard solution; 50  $\mu$ l. of C<sup>14</sup>-lysine-tRNA (3230 counts/minute) solution, made from lysine-tRNA prepared by Dr. G. R. Julian according to a modified method similar to that used earlier (Pestka, 1966b); 100  $\mu$ l. of buffer N-; 540  $\mu$ g. of E. coli ribosomes (in 25  $\mu$ l. of solution); 50  $\mu$ l. of standard Rose Bengal solution; and 3  $\mu$ l. of poly A (if required).

The tubes initially contained dye, ribosomes, and buffer N-. Photooxidation was carried out, and a solution composed of GTP and C<sup>14</sup>-tRNA was added to each tube. Finally, poly A was added, each tube was stirred, and an incubation at 25° C. was carried out for 10 minutes. At the end of the incubation, 0.08  $\mu$ g. of ribonuclease was added to each tube, and the incubation continued for an additional 5 minutes. The reaction was terminated by the addition of 6 ml. of cold 10% TCA solution.

Analysis of binding. The contents of each reaction tube was filtered using the filtration apparatus described under plating and counting procedures, washed seven times with 3 ml. of 5% TCA solution, and the filters containing the  $C^{14}$ -tRNA bound to the ribosomes were dried. The filters were mounted to ring-and-disc sets, and counted as described elsewhere.

Chromatographic procedure for  $C^{14}$ -lysine peptides

The  $C^{14}$ -lysine peptides produced in poly A-directed in vitro incorporation experiments were chromatographed on carboxymethylcellulose (CMC) as described previously (Stewart, 1962; Smith, 1963; Julian, 1965).

Preparation of the CMC column. Carboxymethylcellulose (Cellex-CM, 0.64 meq./g.; Bio-Rad Corporation) was dispersed in g. d. water to make a thick slurry, and poured into a 50 x 1 cm. glass column. The CMC was allowed to settle under the influence of gravity to a height of 40 - 44 cm., washed with 2.0 M ammonium acetate, and rinsed with water.

Preparation of samples. The reaction mixture to be analyzed, contained in a 12-ml. conical centrifuge tube, was thawed, 2 ml. g. d. water were added, and the tube was heated in a boiling water bath for 2 minutes. After cooling, the contents of the tube were transferred by Pasteur pipette to a Pyrex Microanalysis Filter Holder equipped with a 23.5 mm. Millipore filter (0.45  $\mu$  pore size), arranged such that the filtrate could be collected in a graduated tube. Following transfer of the filtrate to the graduated tube, the filter was washed with

one milliliter of lysine solution (1 mg./ml.); 0.5 ml. of ammonium acetate solution (2.0 M); and finally rinsed with g. d. water until a filtrate volume of 10.0 ml. was attained.

The filtrate was carefully mixed, 0.5 ml. was removed for  $C^{14}$ -recovery calculations, and the remaining 9.5 ml. was applied to the washed CMC column. Following application of the sample, the column was washed with 15 ml. g. d. water, 2 ml. of polylysine hydrolysate solution (made by adding 0.05 ml. of standard polylysine hydrolysate to 2 ml. g. d. water), and 5 ml. g. d. water, in the order listed. The exponential salt gradient was started at this time and used throughout the chromatography.

Preparation of the exponential salt gradient. As discussed by Julian (1965b), substitution of ammonium acetate for sodium chloride in the formation of the salt gradient allowed de-salting by lyophilization of the collected fractions. This facilitated further identification by thin-layer chromatographic techniques.

The gradient was prepared by introducing a 1.0 M ammonium acetate solution from a reservoir into a mixing vessel containing 500 ml. g. d. water. The mixing vessel was connected by air-tight fittings to the CMC column, and the apparatus arranged so that the volume of the mixing vessel remained constant over the course of the run.

Collection and radiochemical analysis of CMC peaks

The eluent from the CMC column was pumped by a Buchler

polystatic pump operating at 10 - 15 ml./hour to a scintillation flow-cell (Nuclear-Chicago) mounted in a scintillation detection system (Nuclear-Chicago Liquid Scintillation System Model 6825 equipped with automatic data read-out), and finally to a collection point. Radioactive peaks were monitored by an analytical count ratemeter (Nuclear-Chicago Model 1620B) attached to a rectilinear galvanometric recorder (1.0 milli-ampere; Texas Instruments). Automatic sample collection was accomplished through the use of an LKB fraction collector-distributor (Model 3402B) and 240-sample turntable tied to the Model 6825 Liquid Scintillation System such that 10-minute samples were counted and collected.

#### Thin-layer chromatography of CMC peaks

Each radioactive fraction from the CMC column was collected, lyophilized, and taken up in 0.2 ml. of g. d. water. Five microliter spots were made on thin-layer plates of microcrystalline cellulose (Eastman 6062 plates), and development was carried out with the use of a solvent described by Waley and Watson (Waley, 1953) composed of butanol, acetic acid, water, and pyridine in the ratio 30:6:24:20. Autoradiography of the developed spots was carried out for 5 - 7 days using medical X-ray film (Eastman). Known standards used in conjunction with thin-layer chromatography and autoradiography included C<sup>14</sup>-lysine (normally added to the incorporation mixture), lysyl-lysine hydrobromide (Miles Laboratories), and standard poly-lysine hydrolysate.

Preparation of standard polylysine hydrolysate

Twenty milligrams of poly-L-lysine hydrobromide (Nutritional Biochemical Corporation; Mann Research Laboratories, Incorporated) were hydrolyzed for 80 minutes at 90° C. with 6 ml. g. d. hydrochloric acid in a 12-ml. conical centrifuge tube placed in a heating block. The resulting hydrolysate was lyophilized, washed with one milliliter of ammonium acetate solution (0.1 M), relyophilized, and finally taken up in one milliliter of g. d. water. The resulting 20 mg./ml. solution was stored frozen at -10° C. until use.

## EXPERIMENTAL RESULTS

### The photodynamic effect of Rose Bengal upon bacterial ribosomes

The photodynamic effect upon Escherichia coli ribosomes of a reaction sensitized by the xanthene dye Rose Bengal is illustrated in Figure 6. It can be seen from the graph that ribosomes very rapidly lose the ability to function in poly A-directed synthesis of polylysine in vitro. The results of control experiments performed on this system (Table II) demonstrated that the presence of dye with no exposure to light, or exposure to light in the absence of dye produced no loss of ribosomal activity.

TABLE II. SENSITIZER CONTROL EXPERIMENTS.

<u>Reaction Conditions</u>	<u>Corrected Counts</u>	<u>% Activity</u>
Normal	7103	100
Presence of Dye (no light)	7056	99
Presence of Light (no dye)	7092	100
Presence of dye and light	2181	31

Corrected counts are counts per minute less background -- the counts per minute from an incorporation less poly A (213 counts per minute in this case). Light duration was 60 seconds.

The effect of dye concentration. The photo-inactivation process was sensitive to changes in the dye concentration, as illustrated by the data presented in Table III. As a result of these observations, most of the investigative work was carried out using a dye concentration of 0.2  $\mu$ moles of dye per tube.

The effect of light duration. Figure 6 displays data which demonstrate that the photodynamic effect of Rose Bengal upon bacterial ribosomes was linear with respect to short

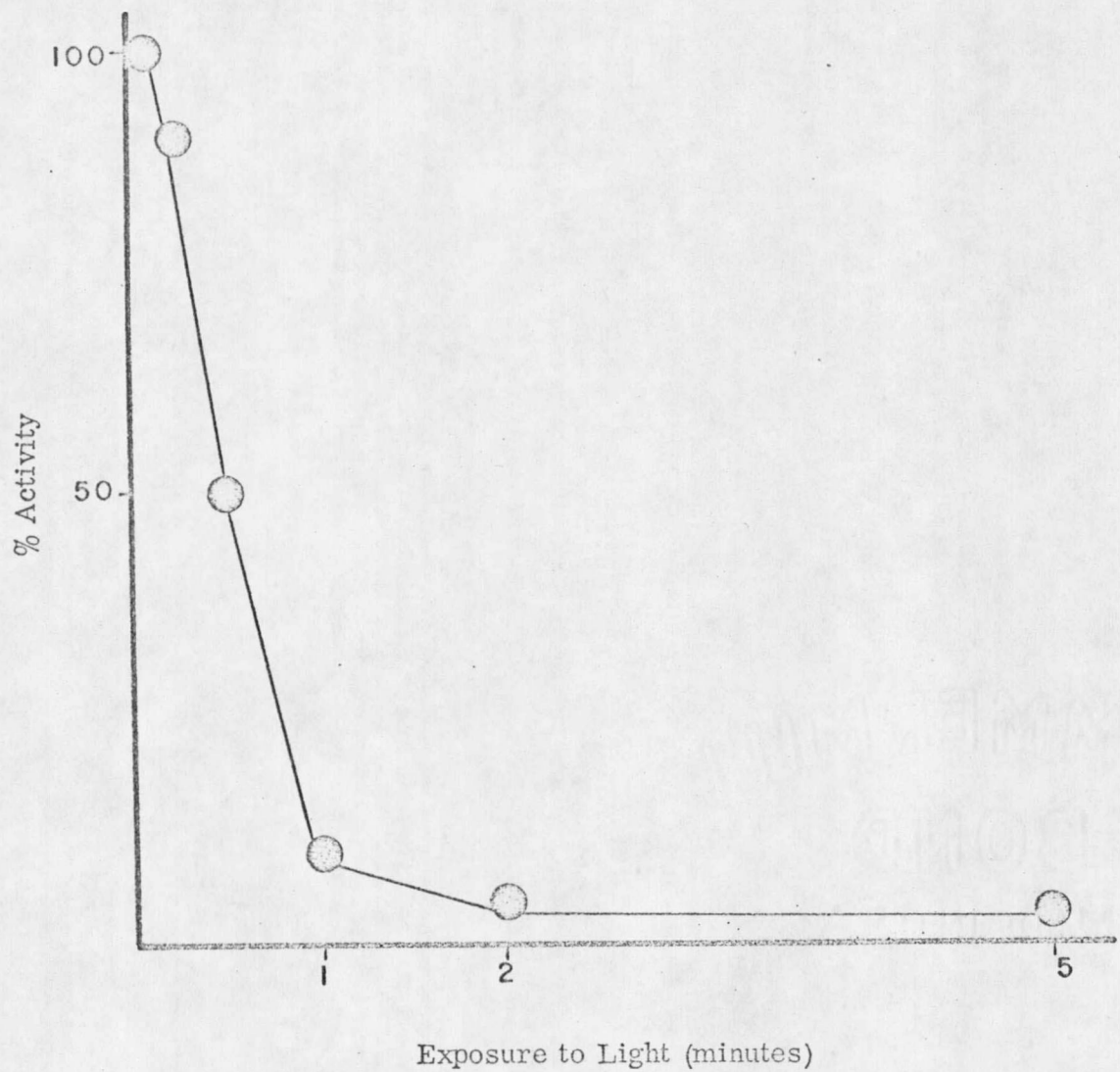


Figure 6. The photodynamic effect of Rose Bengal on Escherichia coli ribosomes. The values plotted were obtained from duplicate averages. Each trial tube contained 625  $\mu$ g Q-13 ribosomes and 0.2  $\mu$ mole of Rose Bengal in 0.45 ml buffer N-.

TABLE III. THE EFFECT OF DYE CONCENTRATION.

<u>Reaction Conditions</u>	<u>Corrected Counts</u>	<u>% Activity</u>
Normal	1036	100
N Dye with Light	275	27
N/2 Dye with Light	492	47
N/4 Dye with Light	654	63
N/8 Dye with Light	954	92

Background was 136 counts per minute. N (normal dye concentration) was 0.2  $\mu$ mole per incorporation tube. Light duration was 60 seconds.

exposure times. To check this observation, a study was made using N/20 dye concentration. The results are presented graphically in Figure 7, and they confirm that the photodynamic effect is linear within the time course of this study.

The effect of ribosome concentration. The data illustrated graphically in Figure 8 show how the observed photodynamic effect depends upon ribosome concentration. At still higher ribosome concentrations (above 1250  $\mu$ g./ml.), the effect shown in Figure 9 is observed. The author has rationalized the activating effect of brief exposure to light at high ribosome concentrations as an indication that the assay system under these conditions was not optimum.

It is concluded in Appendix 1 that the optimum concentration of ribosomes for the poly A-directed incorporation system used as the assay in this investigation was 500 - 600  $\mu$ g./ml. Ribosome concentrations higher than this were inhibitory.

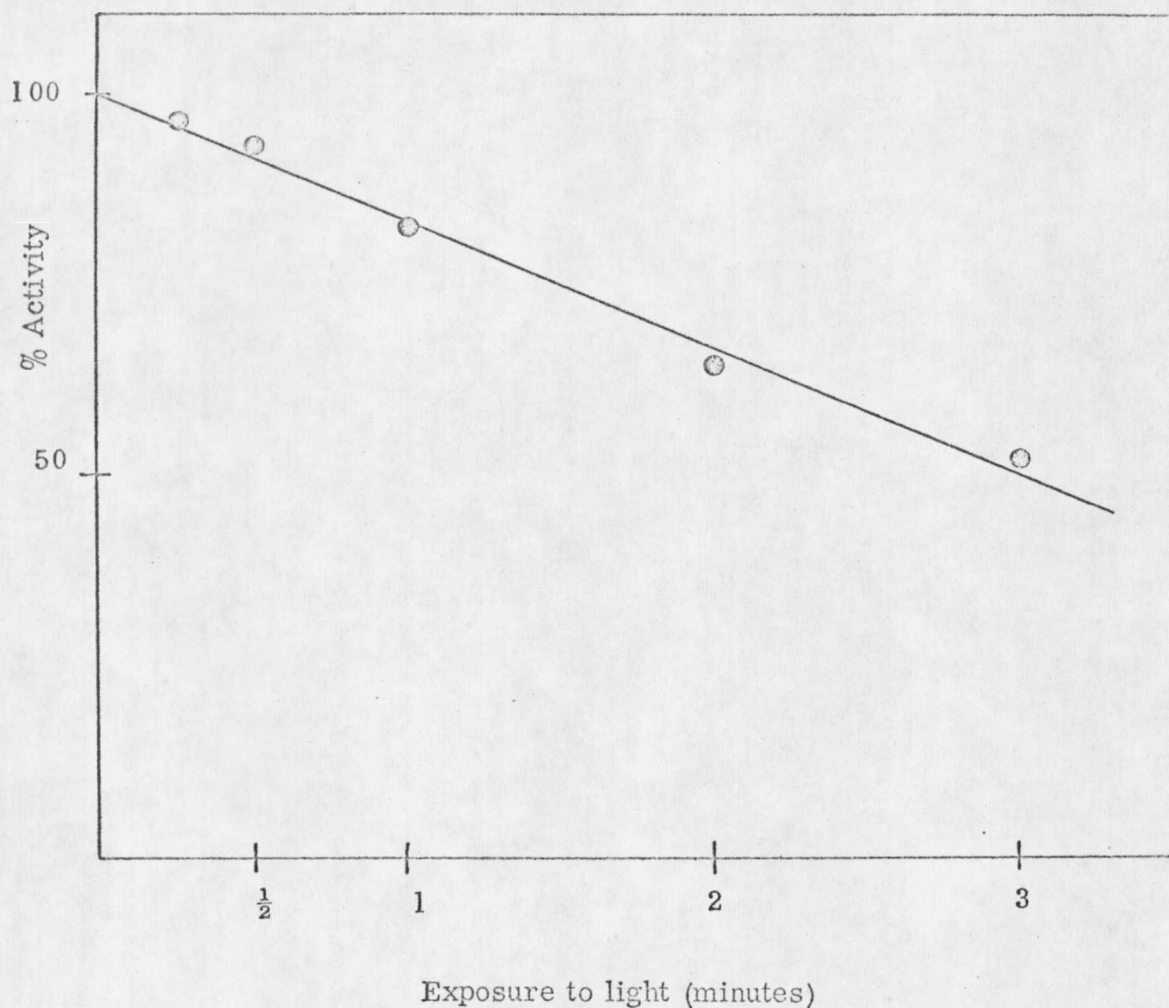


Figure 7. The effect of light duration on the photodynamic inactivation of *Escherichia coli* ribosomes. The values plotted were obtained from duplicate averages. Each trial tube contained 525  $\mu\text{g}$  Q-13 ribosomes and 0.01  $\mu\text{mole}$  of Rose Bengal in 0.45 ml of buffer N-.

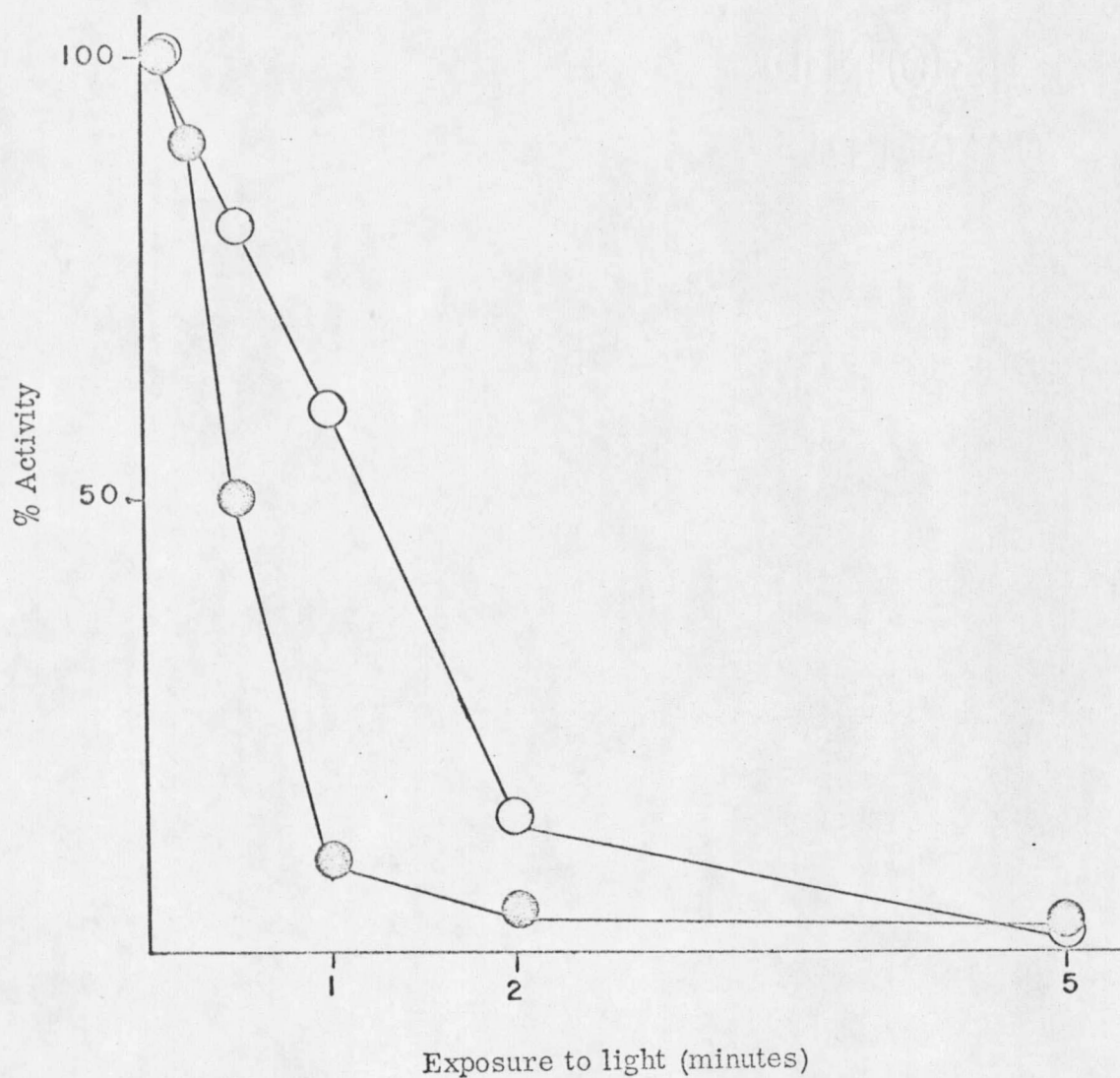


Figure 8. The influence of ribosome concentration on photodynamic inactivation. The values plotted were obtained from duplicate averages. Each trial tube contained 625  $\mu\text{g}$  (● - ●) or 1250  $\mu\text{g}$  (○ - ○) Q-13 ribosomes and 0.2  $\mu\text{mole}$  of Rose Bengal in 0.45 ml of buffer N-.

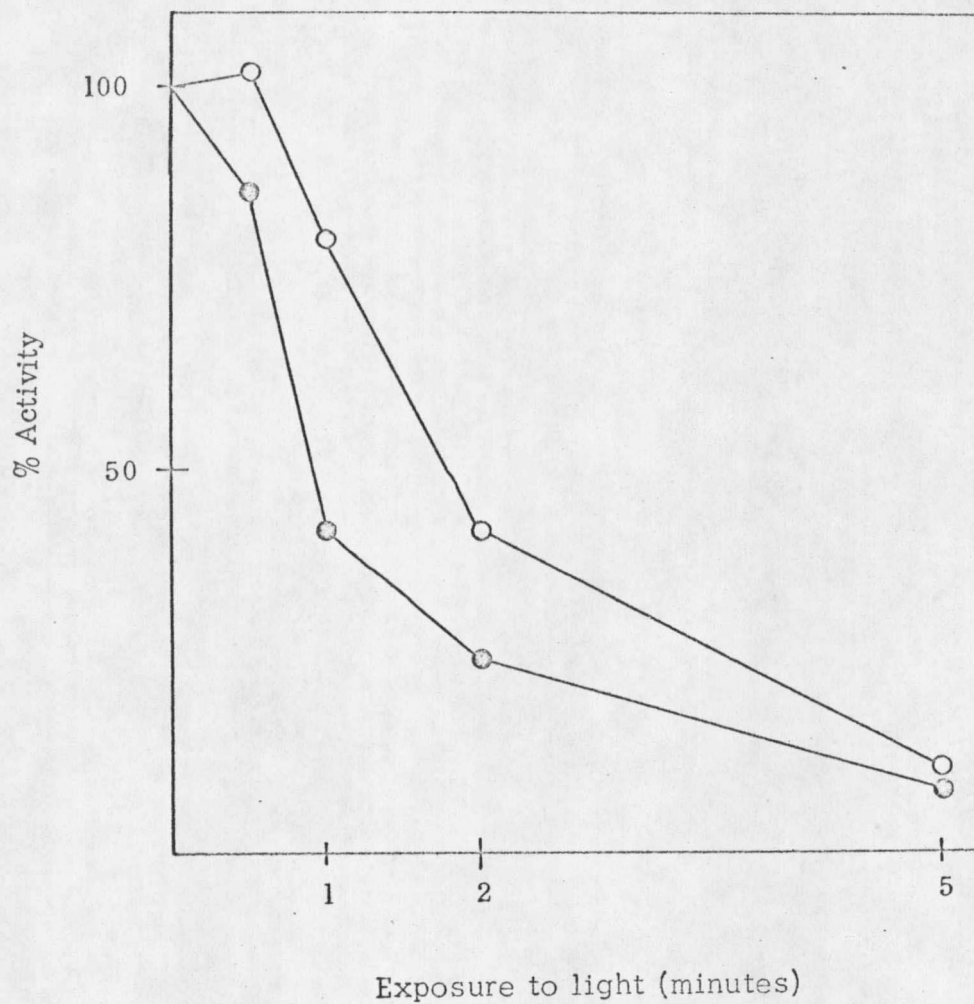


Figure 9. The influence of high ribosome concentration on photo-dynamic inactivation. The value plotted was obtained from duplicate averages. Each trial tube contained 1875  $\mu\text{g}$  (O - O) or 2500  $\mu\text{g}$  (● - ●) Q-13 ribosomes and 0.2  $\mu\text{mole}$  of Rose Bengal in 0.45 ml of buffer N-.

Upon brief exposure to light, therefore, enough of the excess ribosomes are inactivated so as eventually to restore favorable conditions for the remaining active ones. Exposure to light for a time beyond that favorable to establishment of optimum conditions inactivated the system in a more or less normal fashion.

The effect of light intensity from an approximate point source. Figure 10 illustrates the effect of light intensity. It may be inferred from the approximately linear decrease of activity with the reciprocal of the distance from the light source squared that the photodynamic action of Rose Bengal upon bacterial ribosomes was proportional to light intensity.

The effect of distance from the standard illumination source. Since photodynamic action is proportional to light intensity, distance from the slide projector lens (used as the standard illumination source distance) would be a critical factor for reproducibility if the slide projector had an inefficient collimating lens system. Table IV presents data taken from a study done to determine the effect of distance from the projector lens. The region 8 - 12 centimeters (from the lens) produced identical photodynamic effects, so that "approximately ten centimeters" is an adequate description of the operating distance. It was therefore concluded from this study that the collimating lens system of the projector produced an iso-intense beam in the 8 - 12 cm. region.

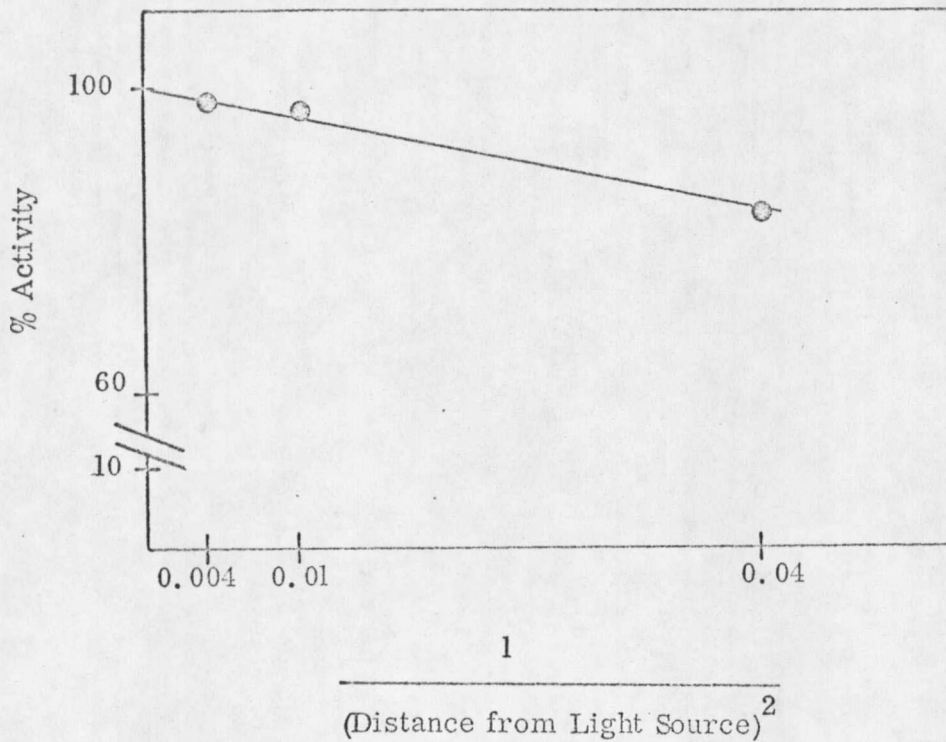


Figure 10. The effect of light intensity on photodynamic inactivation. The value plotted was obtained from duplicate averages. Each trial tube contained 480  $\mu\text{g}$  Q-13 ribosomes and 0.2  $\mu\text{mole}$  of Rose Bengal in 0.45 ml of buffer N-. A 150-watt incandescent bulb was used as an approximate point light source, and each trial tube was positioned at 5, 10, or 15 centimeters from the light bulb for 5 minutes.





































































































