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.

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

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ROBERT THOMAS GARVIN

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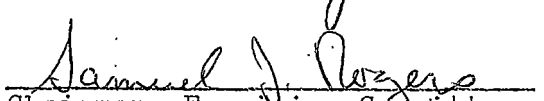
Approved:



Head, Major Department



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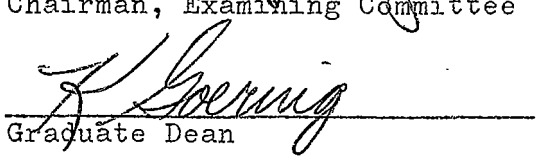

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ABSTRACT

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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

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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).

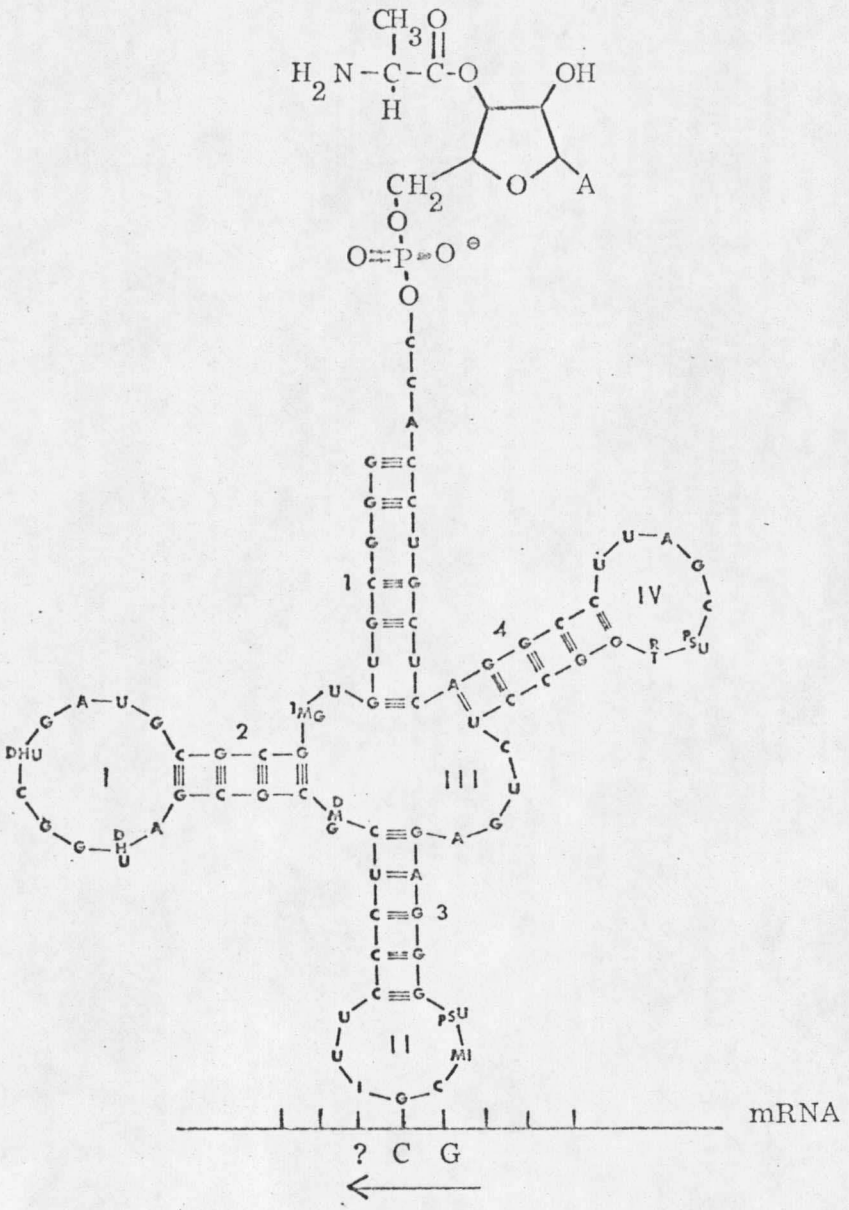

Figure 2. The nucleotide sequence of alanyl-tRNA (from Lipmann, 1969). This sequence was established by Holley and co-workers (1965). At the top of the figure, a sugar-phosphate backbone enlargement has been drawn in, illustrating the manner in which the amino acid is connected to the 3'-hydroxyl of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

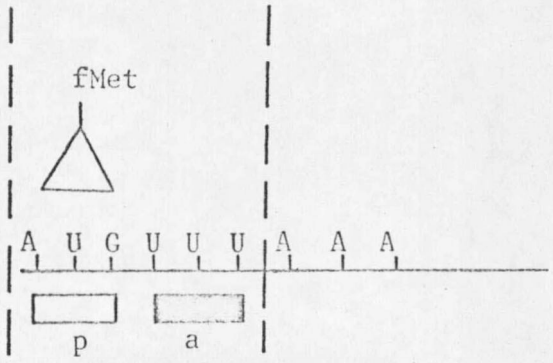

Figure 3. Protein synthesis initiation on the ribosome. In this diagrammatic representation, the 'p' and 'a' sites (the peptidyl tRNA site and the aminoacyl-tRNA site, respectively) shown as blocks represent hypothetical decoding and bond-forming locations. Evidence for the existence of these two sites 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.

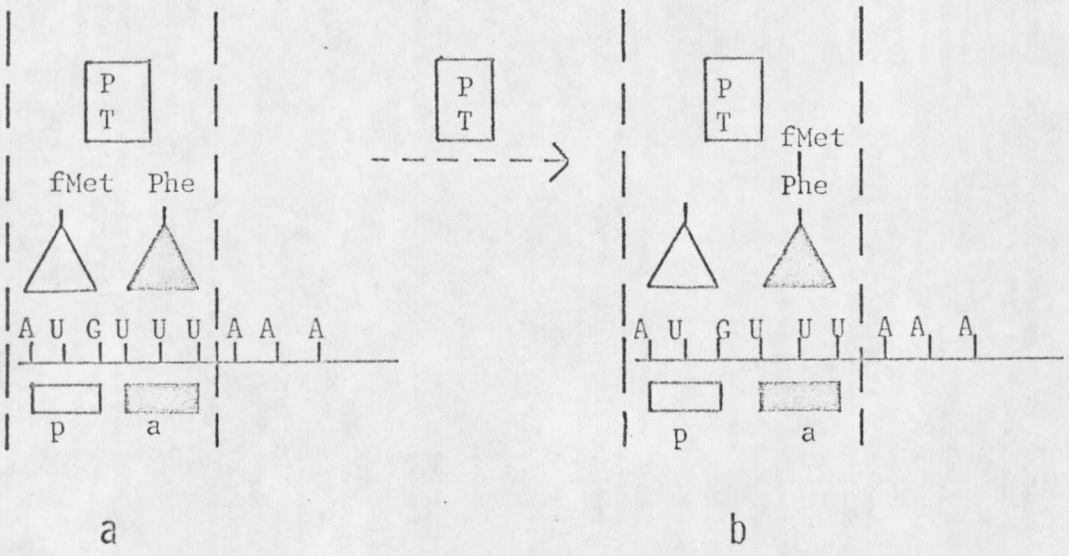

Figure 4. The bond-forming sequence on the ribosome. In this diagrammatic representation, all of the symbols used are the same as those used in Figure 3, with the exception that $\boxed{\begin{matrix}P\\T\end{matrix}}$ represents the peptidyl transferase enzyme located on the 50S particle.

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

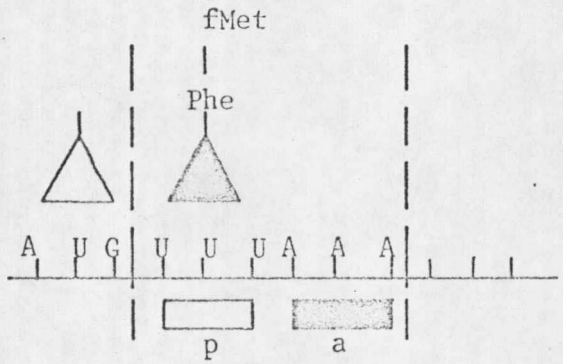

Figure 5. The translocase sequence on the ribosome. This figure is a diagrammatic representation of ribosomal protein synthesis immediately following the translocase operation performed by the ribosome during protein synthesis. All of the symbols used are the same as for Figure 3.

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 greated 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 ($Mg(OAc)_2$); 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 (NH_4Cl); 0.011 M $Mg(OAc)_2$; 0.10 M Tris; pH adjusted to 7.8 with g. d. HCl.

Buffer OB: 0.50 M NH_4Cl; 0.002 M $Mg(OAc)_2$; 0.01 M Tris; pH adjusted to 7.8 with g. d. HCl.

Buffer OC: 0.25 M NH_4Cl; 0.005 M $Mg(OAc)_2$; 0.01 M Tris; pH adjusted to 7.8 with g. d. HCl.

Buffer OD: 0.50 M NH_4Cl; 0.01 M $Mg(OAc)_2$; 0.01 M Tris; pH adjusted to 7.8 with g. d. HCl.

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

Buffer pH 5N: 0.05 M NH_4Cl; 0.011 M $Mg(OAc)_2$; 0.10 M Tris; pH adjusted to 5.2 with g. d. HOAc.

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

Buffer pH 5'N: 0.05 M NH_4Cl; 0.011 M $Mg(OAc)_2$; 0.01 M Tris; pH adjusted to 5.2 with g. d. HOAc.

Pestka and Nirenberg subunit buffer (Pestka, 1966a): 0.0002 M $Mg(OAc)_2$; 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

NH_4Cl; 0.011 M $Mg(OAc)_2$; 0.10 M Tris; pH adjusted to 7.8 with g. d. HCl.

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

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

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

Takanami low-magnesium buffer (Takanami, 1967): 0.01 M Tris; 0.00002 M $Mg(OAc)_2$; 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 μ 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 $Al_2O_3 \cdot 4SiO_2 \cdot H_2O$, 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 thiacyanines 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° 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° 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 mμ) 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^{14}-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^{14}-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 µl. 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 µl. of standard dye solution (which contained 0.2 µmoles of dye); and 20 µl. of ribosomes (approximately 500 µg.) 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^{14}-amino acid (Amersham/Searle; Miles Laboratories) standard solutions plus 100 µl. 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 µmoles ATP; 0.015 µmoles GTP and CTP; 0.25 µmoles PEP; 0.15 E. U. (one microgram) of pyruvate kinase; 0.000415 µmoles of C^{14}-amino acid; 100 µl. of pH 5 enzyme solution; 0.6 mg. of E. coli sRNA; approximately 500 µg. of E. coli ribosomes; 6 µmoles of 2-mercaptoethanol; 0.2 µmoles of dye; and either 50 µg. of poly A (Miles Laboratories) or 25 µg. 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 μ 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 µl. of solution); and 100 µl. of Rose Bengal at the normal concentration (0.2 µmoles of dye). Following photooxidation (carried out in the normal fashion), 0.01 µCi. 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 µl. 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 mμ 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 μl. of C^{14}-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 μl. of buffer N-; 540 μg. of E. coli ribosomes (in 25 μl. of solution); 50 μl. of standard Rose Bengal solution; and 3 μl. 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^{14}-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 μg. 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 μ 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 milliampere; 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^{14}-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.

Reaction Conditions	Corrected Counts	% Activity
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 μmoles 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

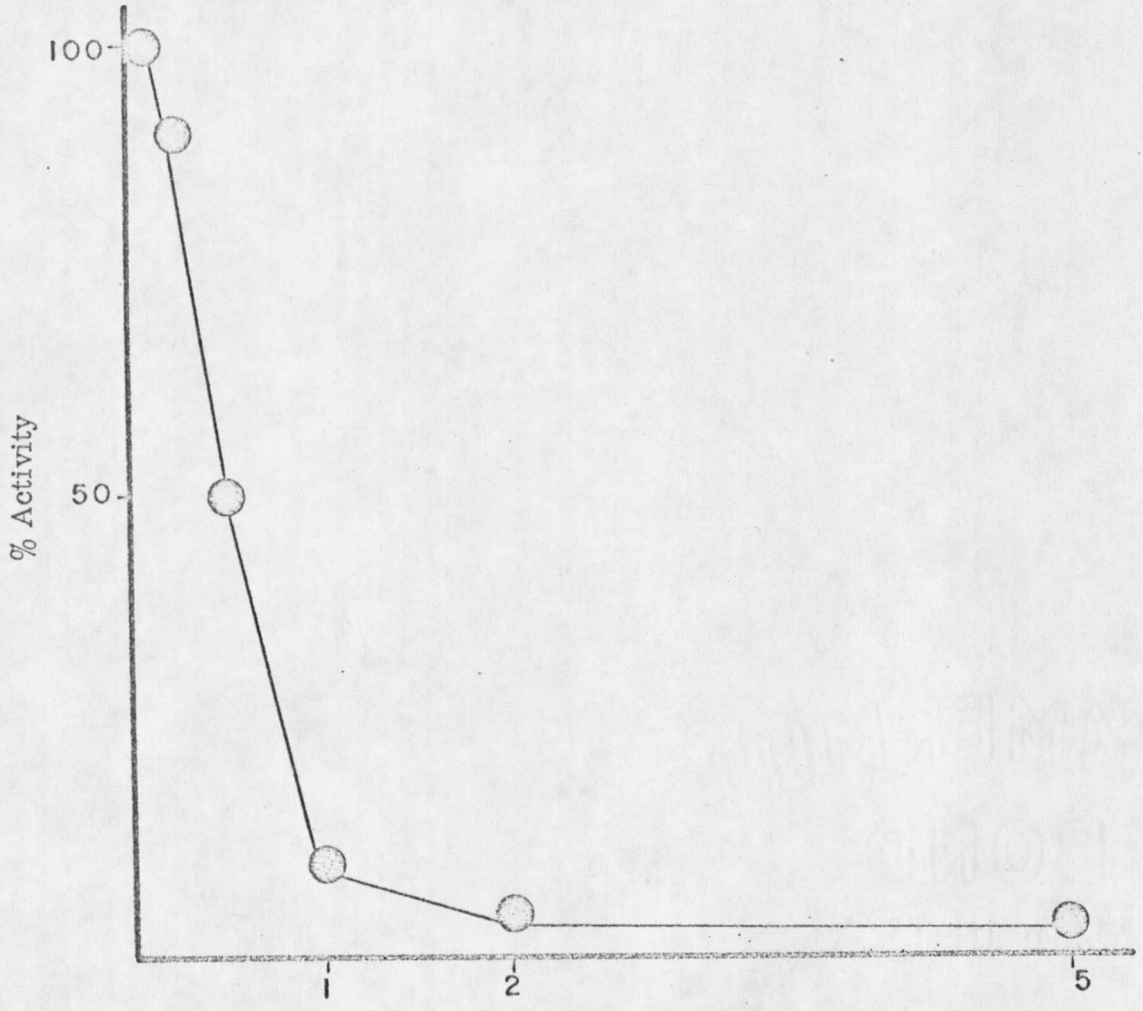

Figure 6. The photodynamic effect of Rose Bengal on Escherichia coli ribosomes. The values plotted were obtained from duplicate averages. Each trial tube contained 625 μg Q-13 ribosomes and 0.2 μmole of Rose Bengal in 0.45 ml buffer N-.

TABLE III. THE EFFECT OF DYE CONCENTRATION.

Reaction Conditions	Corrected Counts	% Activity
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 μmole 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 µg./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 µg./ml. Ribosome concentrations higher than this were inhibitory.

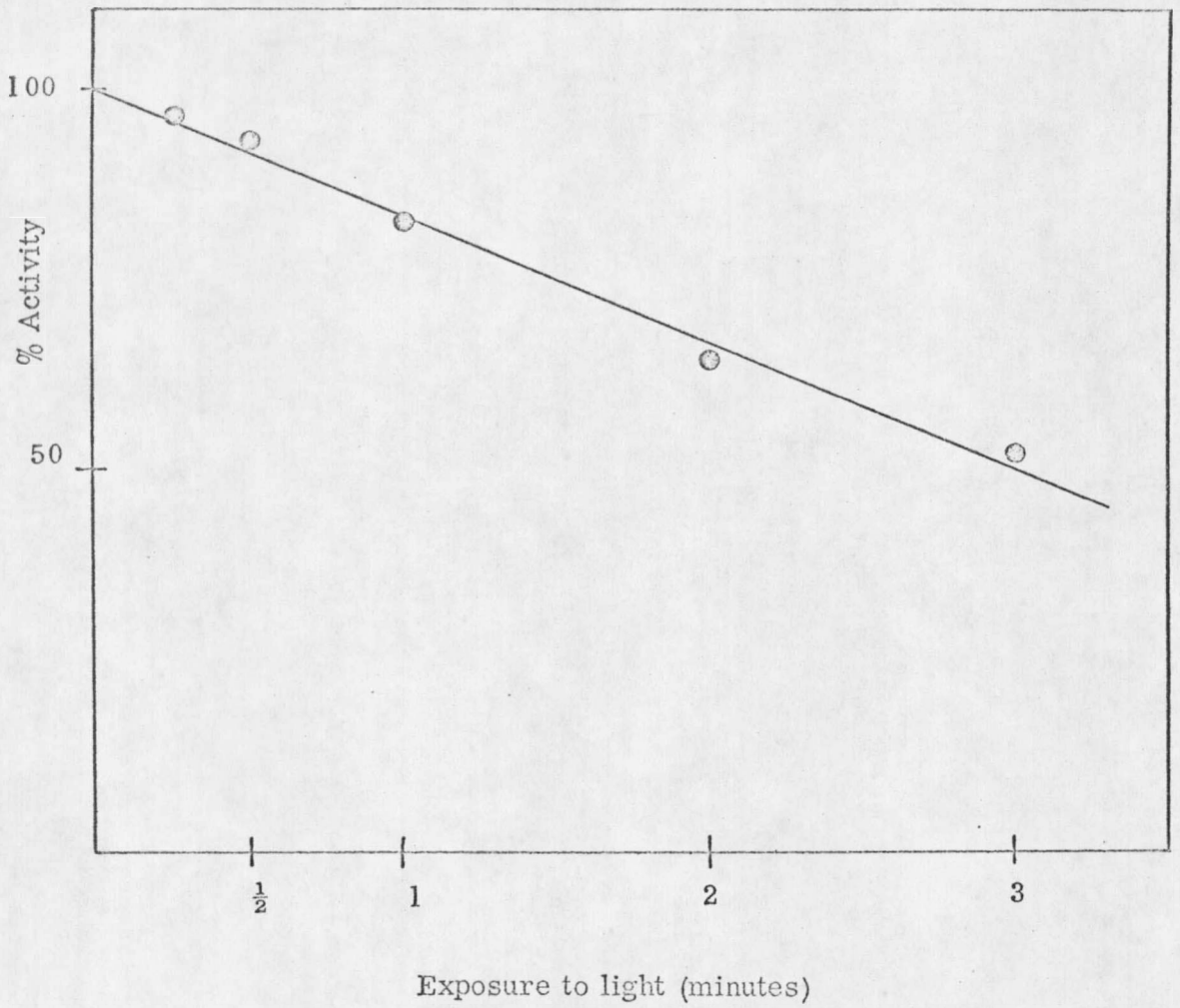

Figure 7. The effect of light duration on the photodynamic inactivation of Escherichia coli ribosomes. The values plotted were obtained from duplicate averages. Each trial tube contained 525 μg Q-13 ribosomes and 0.01 μmole of Rose Bengal in 0.45 ml of buffer N-.

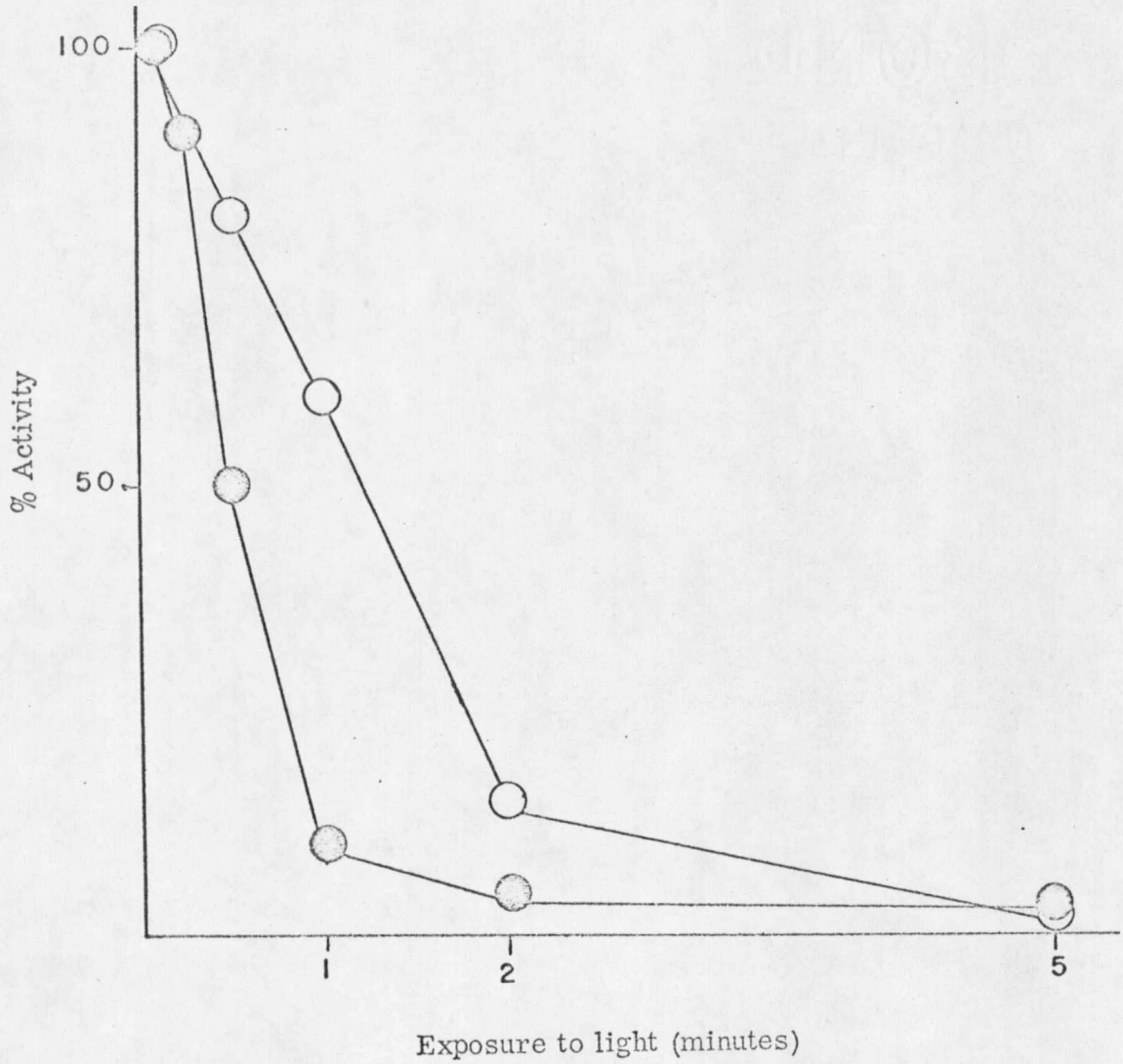

Figure 8. The influence of ribosome concentration on photodynamic inactivation. The values plotted were obtained from duplicate averages. Each trial tube contained 625 μg (● - ●) or 1250 μg (○ - ○) Q-13 ribosomes and 0.2 μmole of Rose Bengal in 0.45 ml of buffer N-.

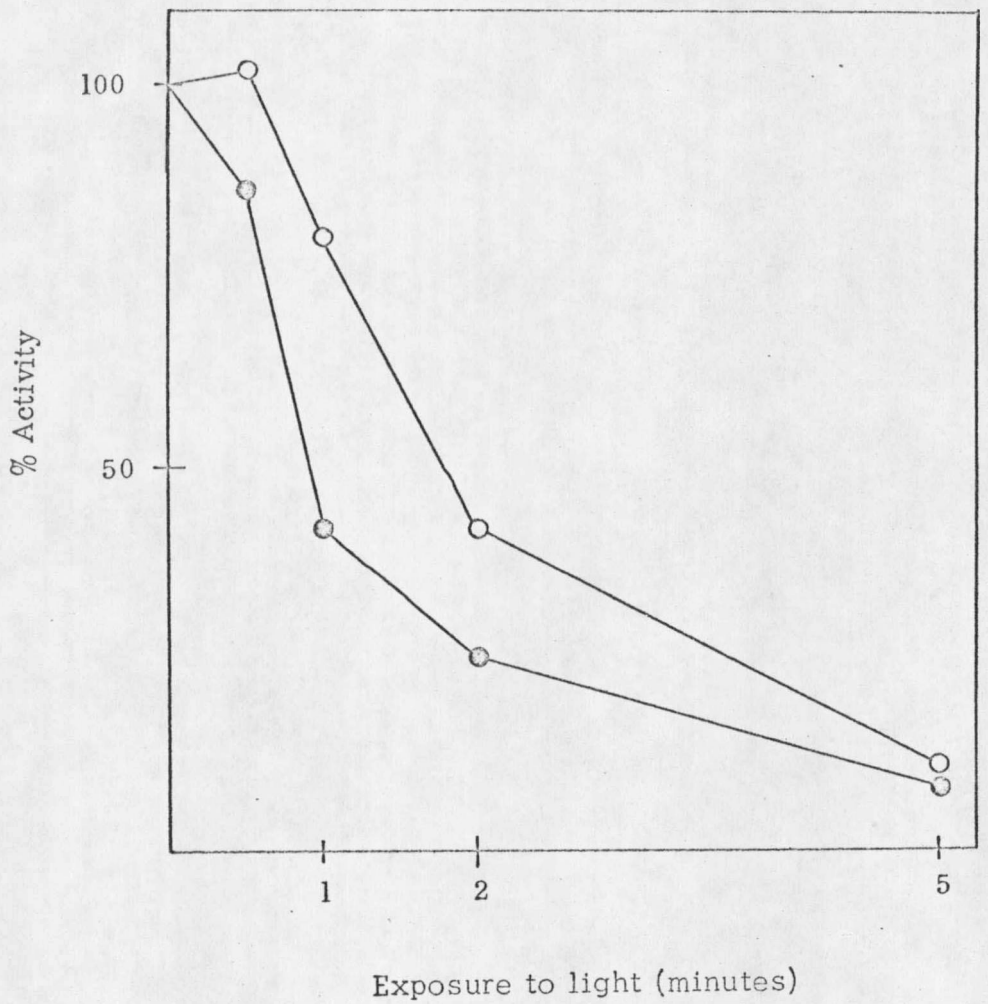

Figure 9. The influence of high ribosome concentration on photodynamic inactivation. The value plotted was obtained from duplicate averages. Each trial tube contained 1875 μg (0 - 0) or 2500 μg (0 - 0) Q-13 ribosomes and 0.2 μmole of Rose Bengal in 0.45 ml of buffer N-.

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.

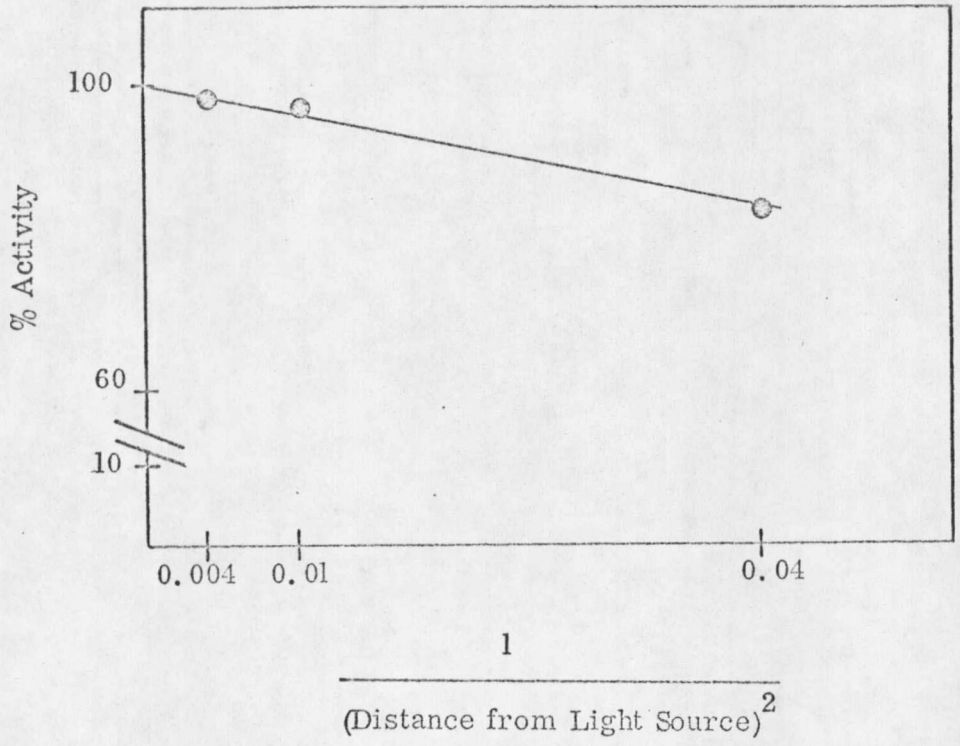

Figure 10. The effect of light intensity on photodynamic inactivation. The value plotted was obtained from duplicate averages. Each trial tube contained 480 μg Q-13 ribosomes and 0.2 μmole of Rose Bengal in 0.45 ml of buffer N-. A 150-watt incandescent bulb was used as an approximate point light source,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

TABLE IV. THE EFFECT OF DISTANCE FROM THE PROJECTOR LENS.

Reaction Conditions	Corrected Counts	% Activity
Normal	2234	100
12 Centimeters from Lens	474	21
10 Centimeters from Lens	481	22
8 Centimeters from Lens	514	23

Background was 152 counts per minute. Dye concentration was 2N. Light duration was 60 seconds.

The effect of 2-mercaptoethanol on photodynamic action. Attempts to show a direct dependence on the presence of oxygen failed. This failure was a consequence of the fragility of the ribosome to aeration methods, making normal oxygen removal and monitoring techniques inappropriate.

The presence of 2-mercaptoethanol in the buffered ribosomal solution during the light exposure period had, however, a marked effect upon photodynamic efficiency. This effect, shown by the data contained in Table V, is an indirect indication that oxygen was required for the full expression of photodynamic action. This conclusion agrees with one established previously (Robinson, 1963).

The conclusion that oxygen is a requirement for the photodynamic effect, taken together with the observed similarity of the reaction conditions to those reported for the photooxidation of proteins and nucleic acids (Weil, 1965; Simon, 1962), lead to the likely assumption that the dye-sensitized photoinactivation here reported is, in fact, dye-sensitized

TABLE V. THE EFFECT OF 2-MERCAPTOETHANOL ON PHOTOOXIDATION.

Reaction Conditions	Corrected Counts	% Activity
EXPERIMENT 1		
Normal	41,582	100
Concentration of 2-mercaptoethanol:		
6 mM	2,351	6
12 mM	3,974	10
18 mM	5,638	14
24 mM	15,287	37
EXPERIMENT 2		
Normal less 2-mercaptoethanol	40,969	100
Normal less 2-mercaptoethanol	40,937	100
6 mM 2-mercaptoethanol present during 5 min. light	677	2
2-mercaptoethanol absent during 5 min. light	294	1
2-mercaptoethanol absent during 10 min. light	42	0
6 mM 2-mercaptoethanol present during 10 min. light	301	1

The background for Experiment 1 was 871 ; for Experiment 2 it was 526. Light duration for Experiment 1 was 2 minutes.

photooxidation (DSPO).

Analytical ultracentrifuge runs of bacterial ribosomes before and after DSPO

As illustrated by the sedimentation patterns displayed in Figure 11, there is no indication of any conformational change

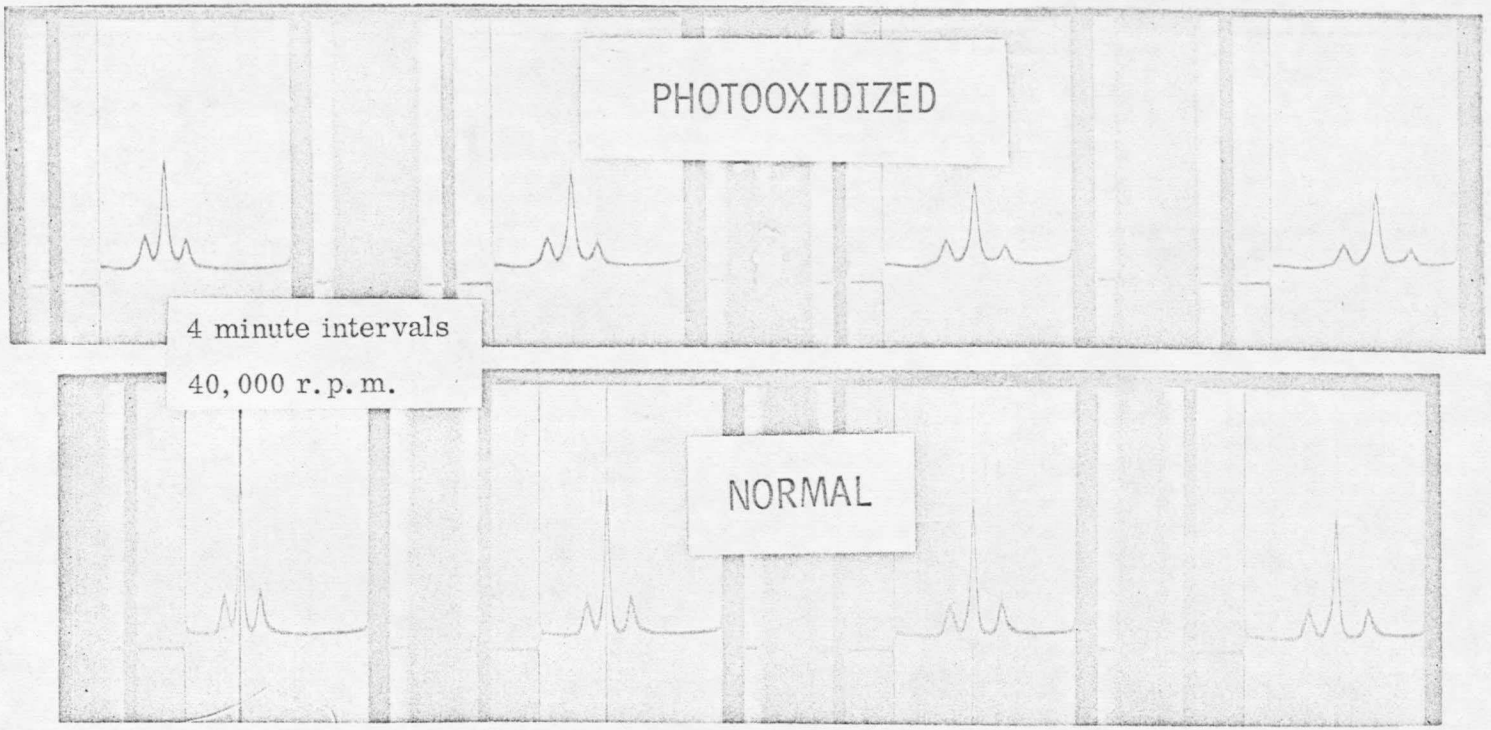

Figure 11. The effect upon sedimentation patterns of Escherichia coli ribosomes following dye-sensitized photooxidation. Each pattern was obtained from 0.6 ml of a solution containing 2.3 mg of Q-13 ribosomes and 2.0 μmole of Rose Bengal in buffer N-. The PHOTOOXIDIZED pattern was obtained from such a solution after an exposure to the light of 10 minutes; the NORMAL pattern was obtained from an identical solution kept in the dark.

in the ribosome after DSPO. This result is consistent with the observations of other investigators (Weil, 1953; Vodrazka, 1959; Ghiron, 1965) that DSPO of certain enzymes, although clearly inactivating them, did not produce any significant alteration of their physical properties.

The differential effect of DSPO on ribosomal subunits

In order to assess which ribosomal subparticle was most affected by DSPO, the particles were separated, and photooxidized separately. One of the best results of such an experiment is presented in Table VI.

TABLE VI. THE EFFECT OF DYE-SENSITIZED PHOTOOXIDATION ON THE SEPARATED RIBOSOMAL SUBUNITS.

Reaction Conditions	Corrected Counts	% Activity
30S + 50S	6248	100
30S	303	5
50S	3608	58
30S* + 50S	3303	53
30S + 50S*	5974	95
30S* + 50S*	2273	36

The background was 606. Dye concentration was normal. Light duration was 60 seconds. A star (*) indicates that the fraction was photooxidized.

The 30S fraction alone did not stimulate the incorporation of lysine, but the 50S fraction alone did. This was interpreted to mean that the 50S fraction was contaminated with 30S particles -- a conclusion borne out by the sedimentation pattern of the 50S fraction shown in Figure 12. Bearing this

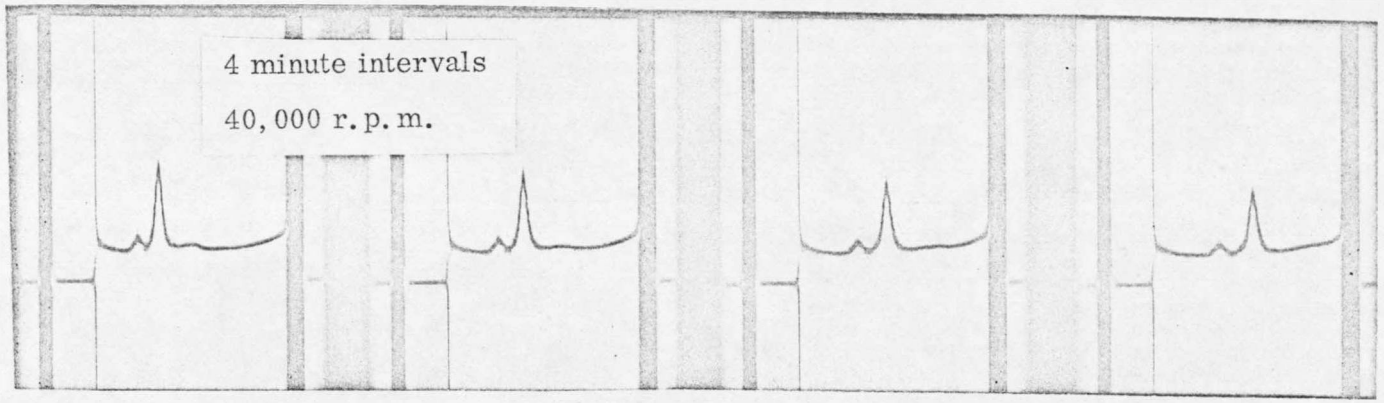

Figure 12. The sedimentation pattern of the 50S fraction used in ribosomal reassociation experiments. This pattern was obtained from 0.6 ml of a solution containing approximately 2.1 mg of ribosomal material (as measured by converting optical density readings to mg of ribosomes).

in mind, the result of photooxidizing the 30S fraction alone (53% activity) was due to the addition of the un-photooxidized 50S fraction (which gave 58% activity alone). Photooxidizing the 50S fraction, then adding the 30S fraction in the dark, restored 95% of the total activity of the system, indicating that the 30S fraction was most photolabile. Although the percentages of inactivation varied from experiment to experiment, the 30S fraction was always the most sensitive to photo-inactivation.

The effect of DSPO on 30S functions

It has been pointed out (Weisblum, 1968) that at least two functions in protein synthesis can be assigned to the 30S subunit. (1) It provides a site of attachment for mRNA (Revel, 1968; Okamoto, 1963). (2) It provides the site for fMet-tRNA binding (at initiation) and subsequent types of aminoacyl-tRNA before peptide bond formation (Nomura, 1967). In addition, however, it is presumed that the mRNA and the 30S subunit are displaced relative to one another during translocation, so that a third function may be tentatively assigned to the 30S pending proof to the contrary. These three functions were investigated in relation to the mode of action of DSPO.

The effect of DSPO on mRNA binding. Binding of mRNA to the ribosome was seriously affected by DSPO as illustrated by Figure 13. Poly U was chosen as the message because of its known affinity for the ribosome. It can be seen that mRNA binding decreases directly with time of light exposure in the presence of Rose Bengal.

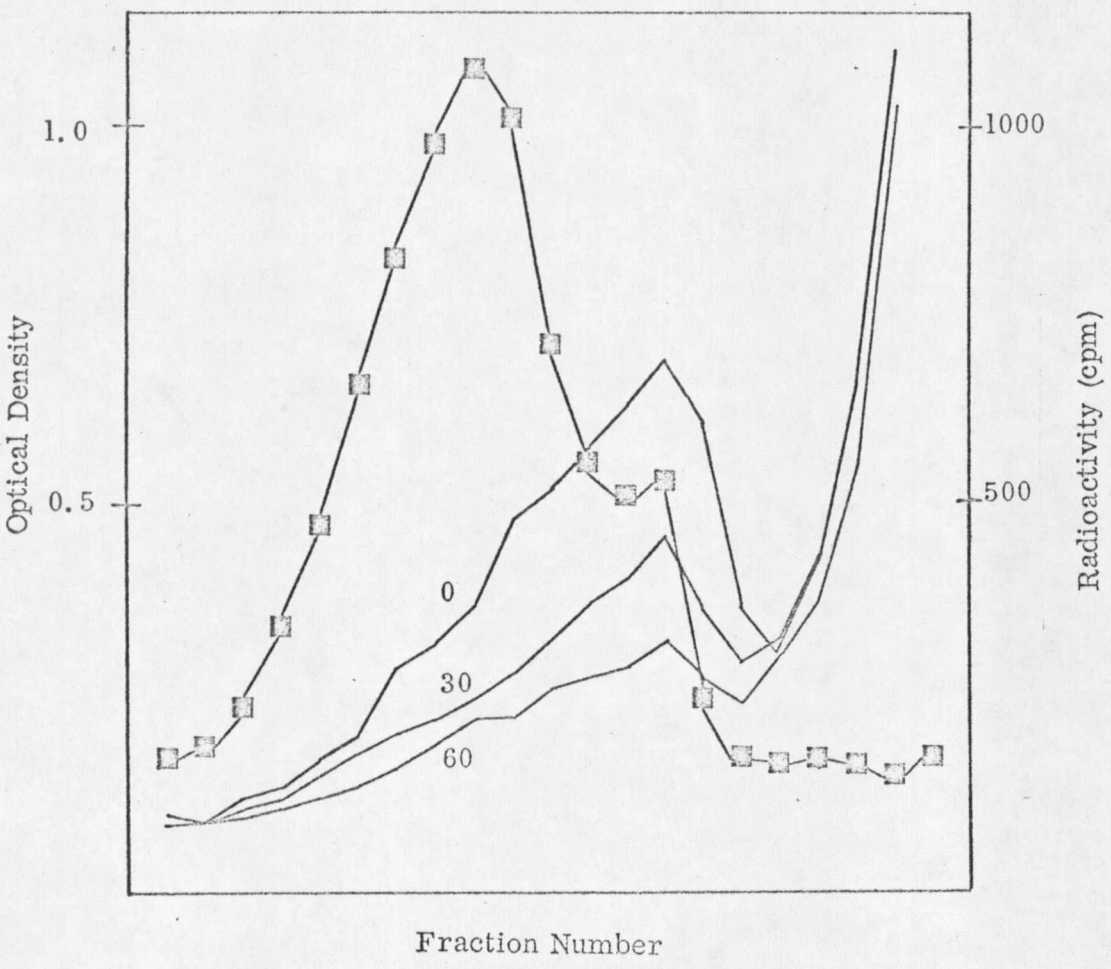

Figure 13. The effect of dye-sensitized photooxidation on mRNA binding. The optical density (▫ - ▫) was measured using an aliquot of the collected 16-drop fraction removed before counting radioactivity (———). The numbers identified with the radioactivity plots are light duration times in seconds.

Protection of the ribosome from DSPO by prior mRNA attachment. Since DSPO affected mRNA attachment, it was reasonable to postulate that incubation of the ribosome with mRNA prior to DSPO might afford protection to the ribosomal site that is susceptible to the effects of photooxidation. This expectation was confirmed for the poly U system by the data presented in Table VII.

TABLE VII. PROTECTION FROM DYE-SENSITIZED PHOTOOXIDATION BY PRIOR mRNA ATTACHMENT.

Reaction Conditions	Corrected Counts	% Activity
Normal	3718	100
Light less Poly U	851	23
Light with Poly U	3920	105
Light on Poly U Only	3717	100

The background was 220. The dye concentration was normal. Light duration was for 60 seconds.

Experiments testing the protection from DSPO offered by prior poly A binding were undertaken, but were inconclusive, possibly due to the known low binding affinity of poly A for the ribosome.

The effect of DSPO on translocation. Early in this investigation, separation of the lysine peptides produced in vitro (by means of chromatographing the incorporation mixture on carboxymethylcellulose) indicated that di-lysine was the only substantial peptide produced after DSPO, since the third peak in Figure 14 was at that time thought to be di-lysine. It can be seen from Figure 15 that three peaks result after

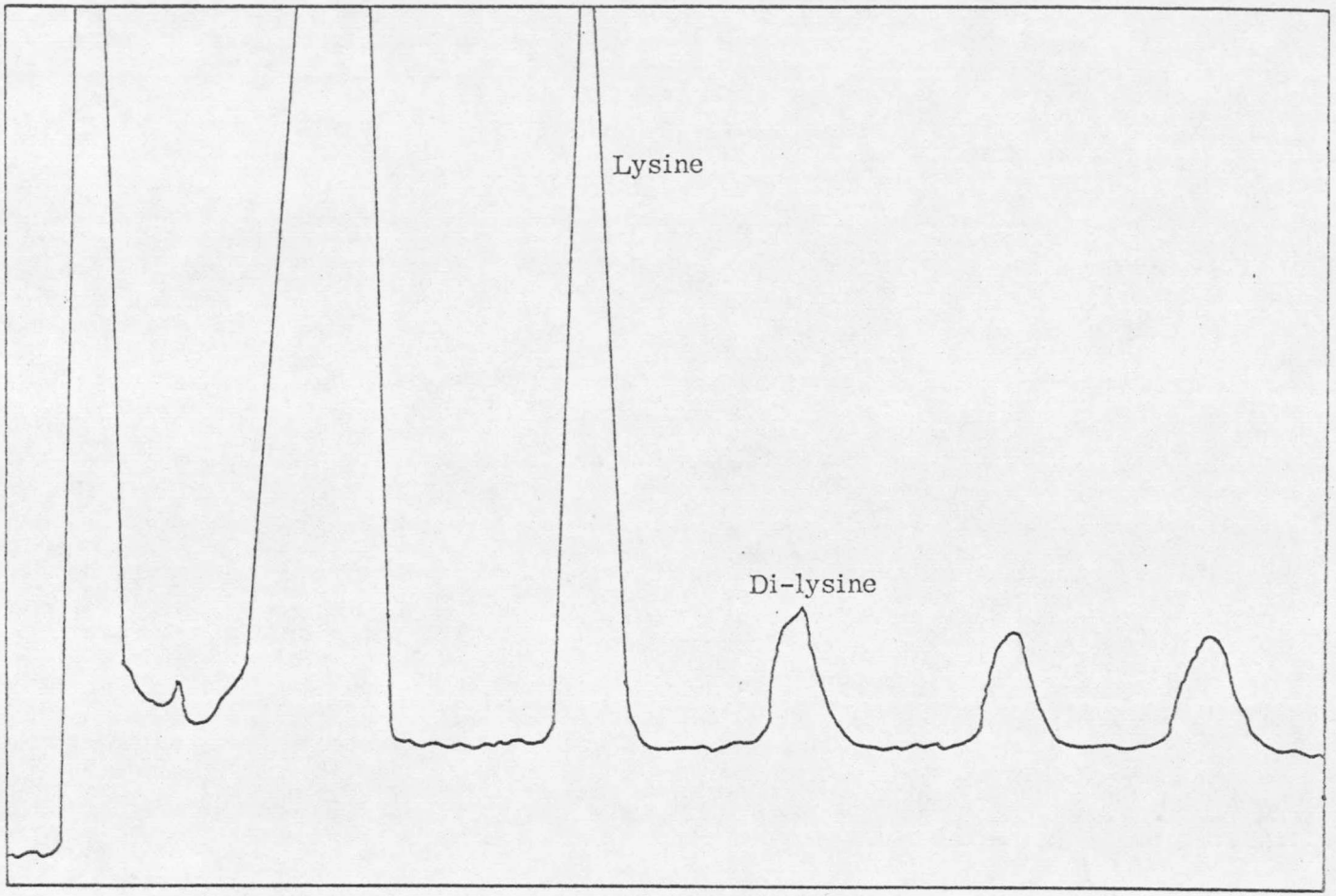

Figure 14. Pattern of lysine peptides from a normal incorporation.

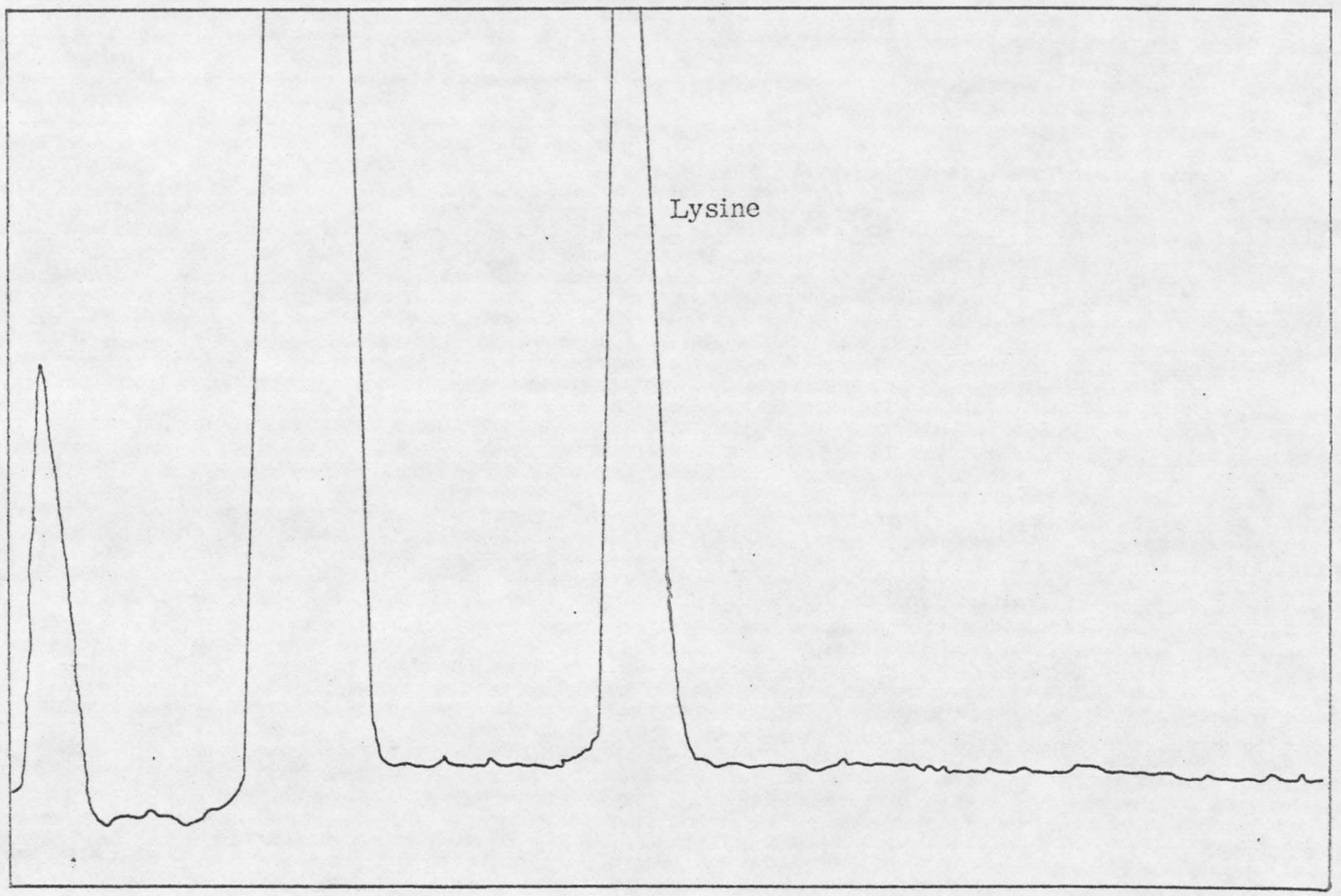

Figure 15. Pattern of lysine peptides obtained from a photooxidized incorporation system.

photooxidation.

If this observation were borne out, it would mean that the translocase function of the 30S particle was being affected by DSPO, because formation of the di-lysine meant that other vital ribosomal functions (mRNA attachment and peptidyl transfer) remained intact.

A series of control experiments was therefore chromatographed in an identical manner to see if the presence of the third peak after photooxidation was an accurate indication of di-lysine. Data from these experiments are displayed in Figures 16 and 17. These profiles are identical to that obtained after DSPO (Figure 15).

Thin-layer chromatography of collected third peaks. Experiments were conducted during which the carboxymethylcellulose third peaks were collected, lyophilized to concentrate and desalt them, and finally analyzed by TLC using microcrystalline cellulose as the support with Waley-Watson solvent for development (Waley, 1953). The results of these experiments show that no significant di-lysine was present after DSPO (see Figure 18).

The effect of DSPO on tRNA binding. Since mRNA binding to the ribosome was affected by DSPO, and tRNA binding has been shown to be dependent upon mRNA attachment to the ribosome (Kaji, 1966), it was presumed that tRNA binding would parallel mRNA binding after DSPO. The results presented in Table VIII confirm this expectation.

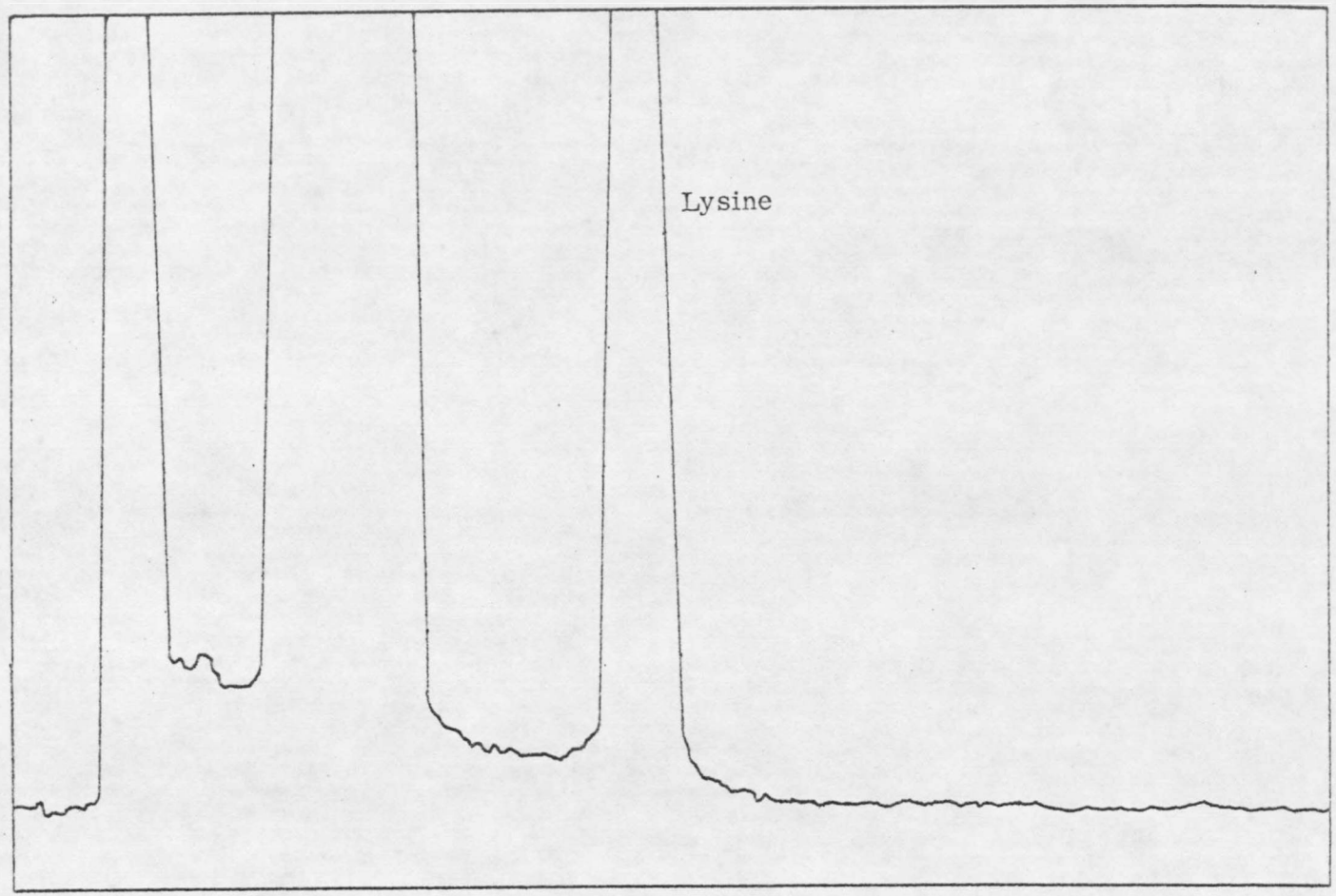

Figure 16. Pattern of lysine peptides obtained from a normal incorporation less poly A.

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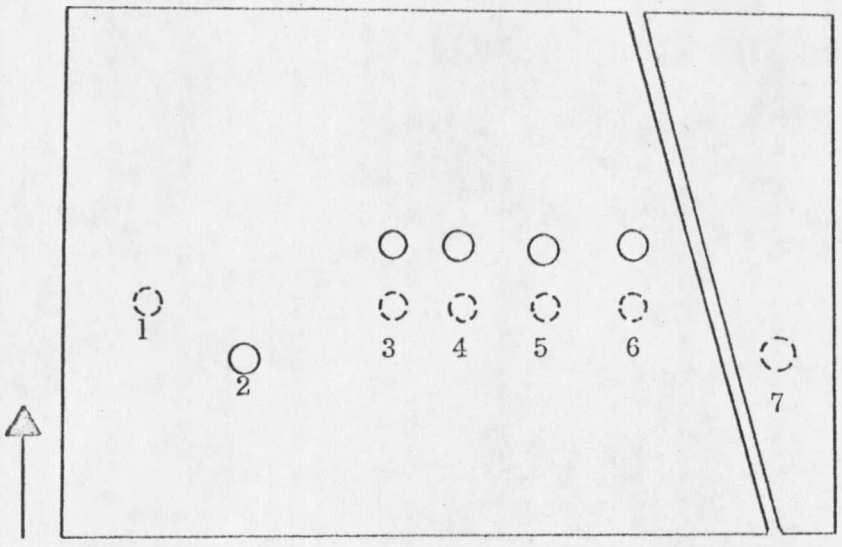


Figure 18. An autoradiogram (- - -) of a thin-layer chromatogram (——) obtained from collected carboxymethylcellulose peaks. Spot 1 was the C^{14}-lysine standard added to the incorporation. Spot 2 was a purchased di-lysine standard. Spots 3, 4, 5, and 6 were collected third peaks from a normal incorporation; an incorporation which contained photooxidized ribosomes; a normal incorporation less poly A; and a normal incorporation less ribosomes, respectively. Spot 7 was the collected fourth peak from a normal incorporation run. This method was used to assign the peak identifications displayed in Figures 14, 15, 16 and 17.

TABLE VIII. THE EFFECT OF DYE-SENSITIZED PHOTOOXIDATION ON tRNA BINDING.

Reaction Conditions	Corrected Counts
Normal	459
Normal less Ribosomes	293
60 Seconds of Light	355
120 Seconds of Light	276

The background was 300. Dye concentration was normal.

Other dyes affecting ribosomal action

Rose Bengal was not the only dye observed to affect ribosomes in the presence of light (although it was the most effective). Table IX presents data for other dyes reported to be effective in biological systems. The marked effect of the thiacyanines is elaborated upon in Appendix 2.

In Table IX, the sensitizing efficiency was based upon the results of a 60 second exposure to light and calculated using the relationship: % loss of activity / % loss of activity using Rose Bengal. All reaction conditions were normal, and the relative sensitizing efficiencies of the dyes were established by several repetitions.

TABLE IX. PHOTOSENSITIZING ABILITY OF SELECTED COMPOUNDS.

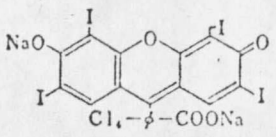
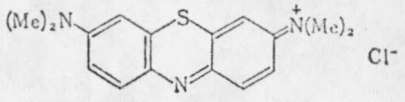
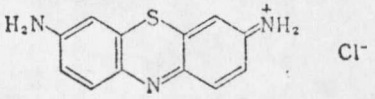
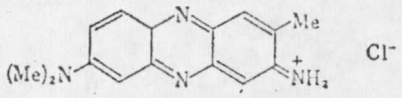
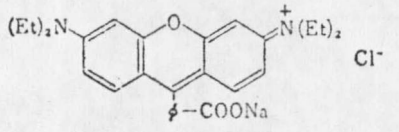
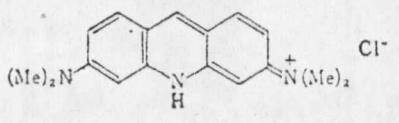
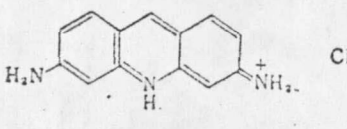
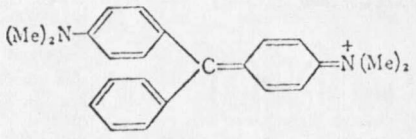
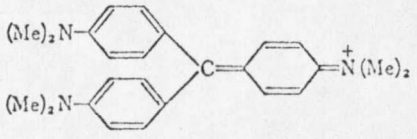
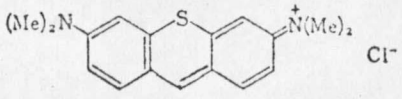
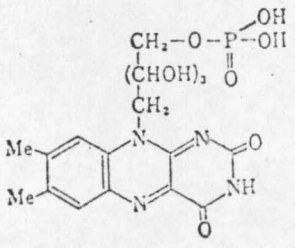
Sensitizer	Structure	Sensitizing Efficiency
Rose Bengal		100
Methylene Blue		33
Thionine		37
Neutral Red		0
Rhodamine B		0
Acridine Orange		0
Proflavin		0

TABLE IX. (Continued).

Sensitizer	Structure	Sensitizing Efficiency
Malachite Green		0
Crystal Violet		0
Thiopyronine		49
FMN		0
Chlorophylline		2

Carbothiacyanines (special dark reaction discussed in Appendix II).

DISCUSSION

Dye-sensitized photooxidation methods have several virtues which could contribute to studying specific components involved in ribosomal function. Typically, in dye-sensitized photooxidation, a substance such as a protein or nucleic acid is exposed simultaneously to a dye and light in the presence of oxygen, causing a chemical alteration of susceptible amino acids or nucleotides. Photo-sensitive moieties include the side chains of histidine, tryptophan, methionine, tyrosine, and cysteine, and the base guanine. Because the dye does not normally have access to internal regions of protein structures, only the surface residues are readily photooxidized (Ray, 1962; Hopkins, 1968). Other advantages of the technique are: (i) the backbone of the polymer (protein or nucleic acid) is not ruptured and the residues affected suffer only minor modifications (Weil, 1953); (ii) harsh reaction conditions are not required (Weil, 1951); (iii) only a few residues are affected (Spikes, 1967); and (iv) some degree of specificity may be derived from the use of different dyes (Bellin, 1968; Bellin, 1965).

It is clear from this study that the principal ribosomal function destroyed by dye-sensitized photooxidation is the mRNA binding capacity of the 30S subparticle. The successful application of classical "substrate-protection" experiments (using poly U) to a biological organelle of this complexity argues eloquently that the ribosomal modification induced by dye-sensitized photooxidation is truly specific.

This study has, however, not produced an equally clear-cut

demonstration as to what specific ribosomal component is involved with mRNA attachment. This fact notwithstanding, a more circuitous route may be taken to the conclusion that the ribosomal component involved is a protein.

Because both protein and RNA can be altered by the process of dye-sensitized photooxidation, advantage can be gained from the known specificity of various sensitizers for the amino acid side-chains of proteins as compared to guanine. Singer and Fraenkel-Conrat (Singer, 1966) found that thiopyronine was approximately 1000-fold more effective than methylene blue for the photooxidation of guanine in RNA from tobacco mosaic virus. Bellin and Yankus (Bellin, 1968) reported that, in general, Rose Bengal was more effective than methylene blue, which in turn was more effective than thiopyronine in the dye-sensitized photooxidation of the five photo-sensitive amino acids. In comparing the ability of various dyes to sensitize the photochemical degredation of deoxyribonucleic acids, Bellin and Grossman (Bellin, 1965) determined that methylene blue and thiopyronine were most effective, wheras Rose Bengal was about ten-fold less effective. In addition, neutral red and acridine orange have been found to be sensitizers for the photooxidation of RNA (Spikes, 1967).

A comparison of these dyes as sensitizers for the photooxidation of Escherichia coli ribosomes (see Table IX) reveals that the relative efficiency of these dyes is roughly similar to the rates found for the photooxidation of histidine, cysteine,

methionine, and tyrosine (Bellin, 1968). The facts: (1) that both methylene blue and thiopyronine are less efficient than Rose Bengal; and (2) that the known RNA sensitizers neutral red, acridine orange, and proflavin have no effect, imply that a protein (or proteins) is (are) the primary substrate(s) being altered by the photooxidation. It is therefore unlikely that guanine residues of the RNA are involved in the inactivation process.

A second argument indicating that the major affected moiety is protein comes from the close correlation of this study of photo-sensitizing efficiency to the results of Gomyo (Gomyo, 1968) on the photo-sensitizing ability of various dyes for the oxidation of histidine. Gomyo found the order of sensitizing efficiency to be: Rose Bengal; methylene blue; thionine; proflavin; and acridine orange (very slight). No effect was observed for Rhodamine B, malachite green, and crystal violet. Except for proflavin (which was found to be inactive in the present study) this order agrees with the order of photo-sensitizing efficiency observed by the author.

Some question remains as to the precise location of ribosomal proteins. The author feels this study indicates that ribosomal proteins (at least those involved with mRNA binding) are located on the surface of the organelle for the following reasons: (1) functional relationships between mRNA, tRNA, and the ribosome are most likely confined to the ribosomal surface owing to the bulk of these constituents; and (2) as

shown above, the dyes used as sensitizers in this study do not normally have access to internal regions of protein structures (Ray, 1962).

These initial photochemical studies on the nature of the bacterial ribosome clearly provide evidence for the critical involvment of a ribosomal surface protein (or proteins) in the mRNA-binding function of the Escherichia coli ribosome. Minor modifications of the photolabile side-chains of the ribosomal protein(s) in situ is sufficient to completely inactivate this essential step in protein synthesis. The rapidity of the inactivation process at low dye concentrations implies an exposed catalytic site -- perhaps a catalytic site analogous to that of ribonuclease, an enzyme which the author feels may have a close similarity to the mRNA-binding function of the 30S ribosomal subparticle, in that both processes initially involve attachment to RNA strands. Ribonuclease was found to be susceptible to dye-sensitized photooxidation (Weil, 1955), and it is known that the active site of this enzyme includes histidine residues (Kenkare, 1966).

The most important conclusion of this study is that specific ribosomal components -- those responsible for mRNA binding -- may be recognized when the organelle is dissociated into its parts. The finding that the ribosomal constituent responsible for mRNA attachment is very likely a protein (or proteins) is intellectually satisfying in the light of the finding by Midgley and McIlreavy (McIlreavy, 1967) that the base content of

rRNA is media-dependent. The author suggests that the function of rRNA is to play a structural rather than a functional role in ribosomal constitution.

APPENDIX

APPENDIX 1

Chromatographic ribosome preparation and small-scale assay methods

Introduction

When this research project was suggested, the successful application of dye-sensitized photooxidation (DSPO) to the study of ribosomal structure could only be attempted if the following conditions were met: (1) the ribosomes to be used in DSPO studies must be pure, i.e., free from the usual supernatant contaminants prevalent in the preparative techniques available at that time; and (2) methods had to be devised either for the large-scale production of pure organelles, or for small-scale assay methods. The author devised techniques which met all of these requirements.

Ribosome preparation

The ribosome preparation was based on a paper on the isolation of ribosomal initiation factors (Salas, 1967). A crude ribosomal preparation was applied to the top of a glass column containing DEAE-cellulose, and a 0 - 1 M ammonium chloride linear gradient was started to elute the ribosomes. After several fractions had been collected, an opalescent fraction was eluted at 0.42 M ammonium chloride. This opalescent fraction proved to be ribosomes, which when pelleted at high speed in an ultracentrifuge gave a perfectly clear, jelly-like pellet.

The current protocol for this preparative technique is presented below under Chromatographic Ribosome Preparation.

CHROMATOGRAPHIC RIBOSOME PREPARATION

-- As of February, 1969 --

PRELIMINARY NOTE: The following procedure is designed to be used with 200 gm of fresh or frozen E. coli cells. ALL glassware, pipettes, tubes, etc. must be ribonuclease-free (as by bentonite washing or the equivalent).

PROCEDURE

STEP #

___ 1. 200 gm of fresh-frozen E. coli cells are mixed with enough cold ribonuclease-free buffer OA* (or N*) to bring the total volume of cells plus buffer to a maximum of 340 ml. 0.6 mg DNAse is added to this mixture, and the mixture is allowed to stand overnight in the 4° cold room.

___ 2. Pass the above through chilled French Pressure Cell at 9,000 psi, and collect in a chilled beaker.

___ 3. If still a little stringy, allow to sit until DNAse has worked.

___ 4. Spin for 45 minutes at 18,000 RPM with Ti-30 head in the Model L. This brings down a solid pellet of cell debris, etc.

___ 5. Pour supernatant into clean tubes and spin again for 120 minutes at 18,000 RPM.

___ 6. Remove top 90% of supernatant with an RNAse-free syringe, being careful not to disturb the glurpy pellet.

___ 7. Centrifuge this supernatant for 180 minutes at 50,000 RPM in the Ti-50 rotor.

___ 8. Remove top 80% of the supernatant with an RNAse-free syringe or pipette, place in a chilled beaker and save. (This is crude S-100.)

___ 9. Add 2 ml buffer OB to the tubes containing the pellets and allow to stand in the 4° cold room for 12 to 14 hours (with occasional stirring).

___ 10. S-100 Fraction (from Step 8): Spin crude S-100 fraction 180 minutes at 50,000 RPM and bottle TOP TWO-THIRDS in 5 ml aliquots. Store in Revco at -90° C.

___ 11. At this point, a decision must be made concerning the pellets obtained in Step 9. If the pellets were small, combine tubes so that you have only half as many as you started with; if the pellets were large, leave as is.

In any case, fill the tubes used with buffer OB and spin for 15 minutes at 25,000 RPM in Ti-50 rotor (low-speed wash).

___ 12. Pour the supernatant into clean tubes, and spin for 180 minutes at 50,000 RPM in Ti-50 rotor.

___ 13. Remove and discard the top 80% of the supernatant fluid.

___ 14. Add 2 ml buffer OC to the tubes containing the pellets, and let dissolve over a period of 4 to 6 hours (with occasional stirring).

NOTE: It may be that the ribosomal pellets at this point are exceptionally dirty. If this is the case, repeat the low-speed wash and re-pellet the ribosomes (Steps 11 through 13).

___ 15. Combine the dissolved ribosomes and adjust the total volume to about 50 ml by adding buffer OC. Take O.D. readings to determine the concentration of this ribosomal solution.

___ 16. Introduce 200 mg of ribosomes onto the column with an RNAse-free syringe (probably about one-third of the ribosomal solution prepared in Step 15), and allow to run until the ribosomes are all on column.

___ 17. Rinse the column top with a small amount of buffer OC, and run this onto the column; now fill the top of the column with buffer OC. Put 500 ml of buffer OC into the tubular delivery bottle and run the buffer through tubing to the column cap. Screw on the cap and adjust the effluent to collect 20 ml fractions (collection time: about 20 minutes/fraction).

___ 18. Fill the top of the column with buffer OD. Put 500 ml of buffer OD into the delivery bottle and set up to run through the column as before. Collect fractions as done previously.

___ 19. Spin down the collected ribosomal fractions (the ones with a bluish tinge) at 50,000 RPM for 4 hours.

___ 20. Remove and discard the top 80% of the supernatant, add 2 ml of buffer OB to the tubes containing the ribosomes, and allow to dissolve at 4° C.

___ 21. Determine concentration of ribosomes by an O.D. measurement. (At 260 mμ, O.D. of 1 equals 60 μg/ml). Dilute first 1:2000 — 16.6 O.D./mg.

___ 22. Adjust to concentration desired, and bottle in appropriate aliquots. Store in Revco (after proper labeling) at -90° C.

NOTE ON ELUTION OF RIBOSOMES: According to Stanley, ribosomes washed with 0.5 M ammonium chloride still contain the initiation factors, while ribosomes washed with 1.0 M ammonium chloride do not. Therefore, a choice exists for the elution buffer:

This protocol was developed in conjunction with a sedimentation study (done in the Beckman Model E analytical ultracentrifuge) of each step. The effect of each manipulation was determined. A preparative technique similar to this one was developed independently by Stanley and Wahba, and has recently been published (Stanley, 1967). The method developed by the author, however, allows isolation of ribosomal material without loss of 30S subparticles.

The quick-prep method. In addition to the standard procedure, a prpearation was worked out which returned active ribosomes in about half the time required for a normal preparation. This procedure is given below under Quick-Prep Modification.

Elution profiles of normal and quick-prep ribosomes. The elution profiles from DEAE-cellulose using displacement chromatography of ribosomes prepared by each method are shown in Figures 1 and 2. With the recent interest in supernatant factors, these profiles could indicate a preparative procedure which returns both ribosomes and ribosomal factors. The protein peaks preceeding the ribosomal elution may be the T and / or G factors -- especially when one considers the method by which these factors have been isolated in the past.

Small-scale assay methods

The assay system used in this investigation was determined by a series of studies on the characteristic variables of the incorporation system for lysine and phenylalanine. The buffers and recipes given in Materials and Methods have been

QUICK PREP MODIFICATION

It has been shown feasible to prepare ribosomes very quickly using a modified column preparation. These quick prep ribosomes were just as active as the long-prep ones; however, the S-100 derived from the quick prep <u>may</u> contain a small amount of ribosomes as contaminant (it also may not, depending on the operator).

The quick prep is normal until step 8.

_____ 8. Remove the top 70% of the supernatant with an RNAase-free syringe or pipette, place in a chilled beaker, and bottle syringe or pipette in 4 - 5 ml aliquots as the S-100 fraction. Store at -90°.

_____ 9. As usual.

_____ 10. Fill the tubes with buffer CC, and spin for 45 minutes at 25,000 RPM in the Ti-50 rotor (low-speed wash).

_____ 11. Decant the supernatant into a chilled beaker, and introduce immediately onto the column.

_____ 12. Continue with step 17 of the normal prep.

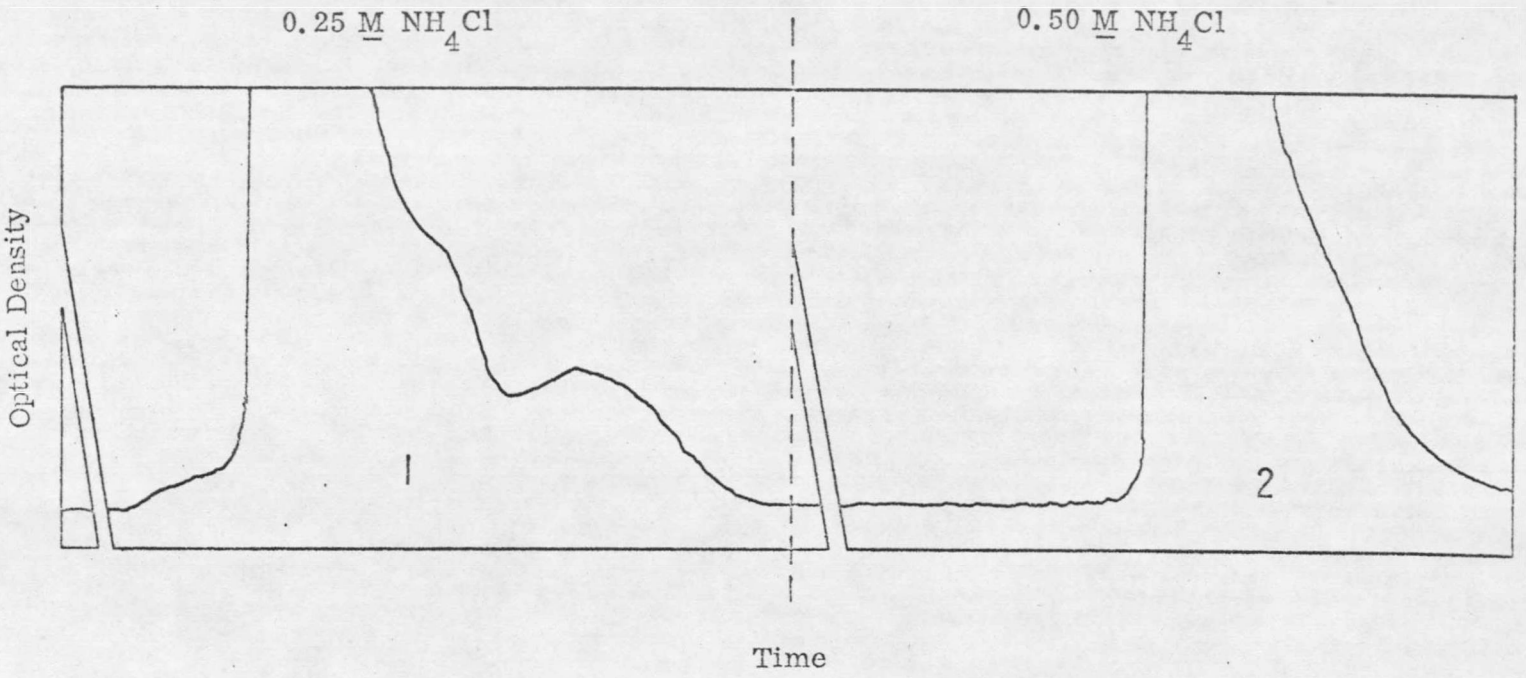


Figure 1. Elution profile from DEAE-cellulose of a normal ribosomal preparation. This profile was obtained from Step 18 of the Chromatographic Ribosome Preparation. Area 1 contained straw-yellow protein material washed off the ribosomes. Area 2 contained the opalescent ribosomal fraction.

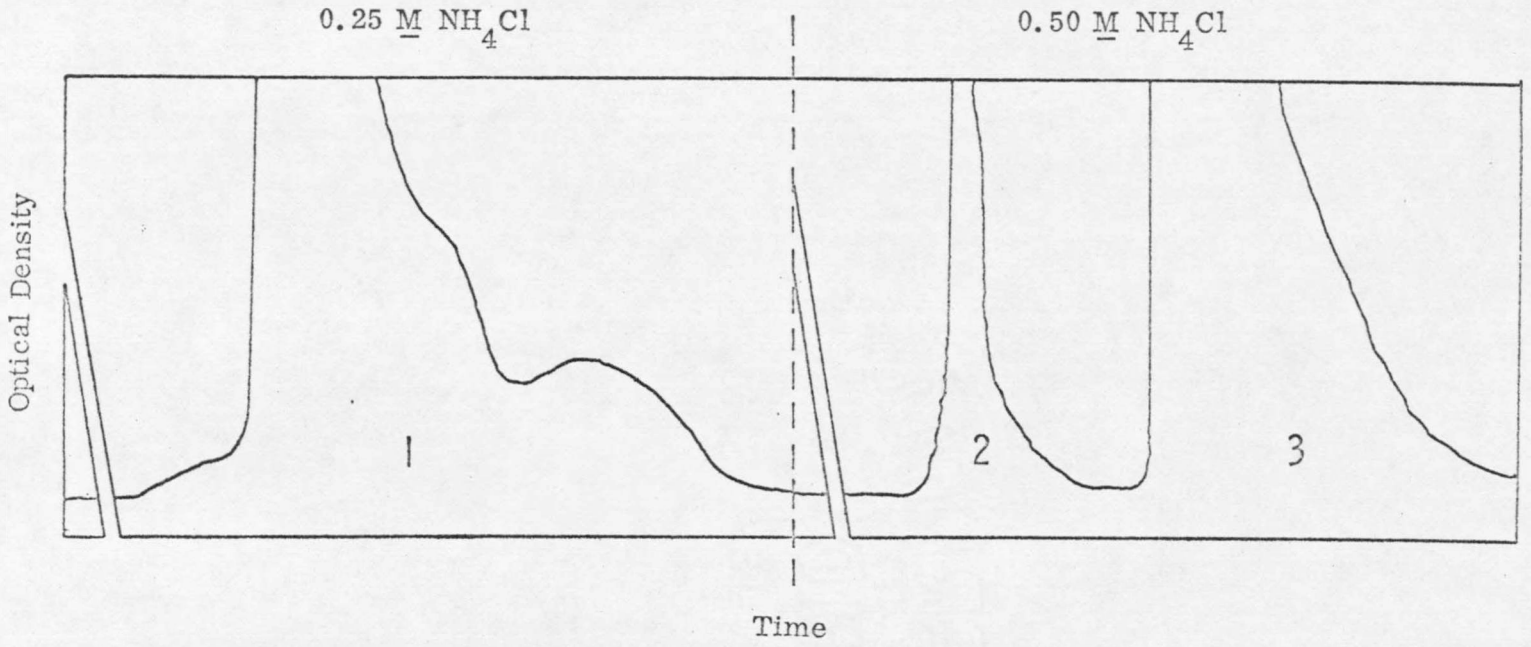


Figure 2. Elution profile from DEAE-cellulose of a modified ribosomal preparation. This profile was obtained from Step 11 of the Quick Prep Modification. Area 1 contained the straw-yellow protein material washed off the ribosomes. Area 2 contained a yellow material, mostly protein. Area 3 contained the opalescent ribosomal fraction.

based on the results of these investigations.

The effect upon incorporation of GTP, CTP and UTP. As shown by the data displayed in Table I, the presence of UTP has slight, if any, effect on the incorporation. The presence of GTP and CTP (studied together), however, did stimulate the system.

TABLE I. THE EFFECT UPON INCORPORATION OF GTP AND CTP, AND UTP.

Reaction Conditions	Corrected Counts	% Activity
EXPERIMENT 1		
Normal	4353	100
Normal less GTP, CTP, UTP	1879	43
EXPERIMENT 2		
Normal	28,465	100
Normal less UTP	27,941	100
Normal less GTP and CTP	24,402	86
Normal less ATP, GTP, CTP, UTP, PEP, pyruvate kinase	144	0

The background for Experiment 1 was 83. The background for Experiment 2 was 265.

This observation agrees with the results of other investigations on in vitro incorporation systems (Nirenberg, 1961; Lucas-Lenard, 1967).

The effect of PEP, pyruvate kinase, and ATP. The enzyme pyruvate kinase and substrate phosphoenolpyruvate (PEP) are put into in vitro incorporation systems in order to maintain the level of ATP (Nirenberg, 1961). This enzyme and substrate together constitute an ATP-generating system, producing ATP

from the products of the aminoacyl ligase reaction ("charging reaction"). Consequently, one would expect that either ATP, or PEP and pyruvate kinase would be necessary for the in vitro incorporation. Table II supports this contention, and the data indicate the most efficient concentration level for the ingredients.

TABLE II. THE EFFECT UPON INCORPORATION OF PEP AND PYRUVATE KINASE.

Reaction Conditions	Corrected Counts	% Activity
EXPERIMENT 1		
Normal	5488	100
Normal with 1/10 PEP and pyruvate kinase	5373	98
EXPERIMENT 2		
Normal	28,465	100
Normal less PEP and pyruvate kinase	17,134	60
Normal less ATP	9,092	31

The background for Experiment 1 was 190; for Experiment 2 it was 265.

The effect of 2-mercaptoethanol. The presence of 2-mercaptoethanol, reported by many investigators to be important to incorporation (Nirenberg, 1961; Miyazawa, 1967), was shown to have a very slight effect in this system, as the data of Table III indicate.

The effect of magnesium ion level on incorporation. Figure 3 is the result of a study done to determine the effect of magnesium ion on incorporation efficiency. It is apparent

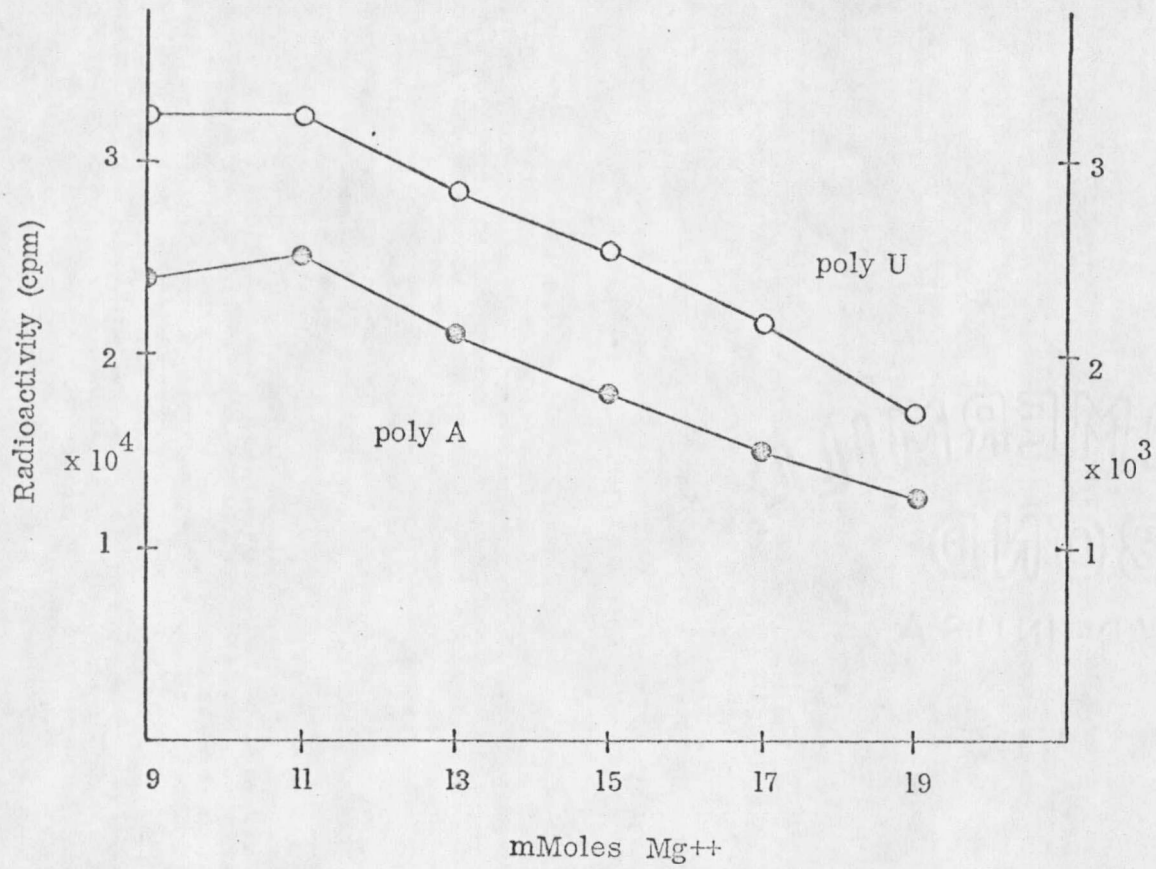


Figure 3. The effect of magnesium ion concentration on incorporation efficiency. This titration was done using the normal incorporation system with 540 μg of ribosomes.

TABLE III. THE EFFECT OF 2-MERCAPTOETHANOL.

Reaction Conditions	Corrected Counts	% Activity
EXPERIMENT 1		
Normal	56,731	100
Normal less 2-Mercaptoethanol	55,960	99
2 x 2-Mercaptoethanol	57,304	100
4 x 2-Mercaptoethanol	54,589	96
EXPERIMENT 2		
Normal	41,495	100
Normal less 2-Mercaptoethanol	41,463	100

The background for Experiment 1 was 1346; for Experiment 2 it was 526.

that the peak effect occurs at about 10 - 12 mM for both lysine and phenylalanine incorporation.

The effect of ammonium and potassium ions on incorporation. Both ammonium and potassium ions work in the lysine incorporation system, as may be inferred from Table IV.

TABLE IV. THE EFFECT UPON INCORPORATION OF AMMONIUM AND POTASSIUM IONS.

Reaction Conditions	Corrected Counts	% Activity
EXPERIMENT 1: EFFECT UPON POLYPHENYLALANINE		
Normal with Potassium Ion	5240	100
Normal with Ammonium Ion	3850	71
EXPERIMENT 2 : EFFECT UPON POLYLYSINE		
Normal with Potassium Ion	14,839	100
Normal with Ammonium Ion	14,697	99

The background for Experiment 1 was 121; for Experiment 2 it was 828.

However, ammonium ion affords a better precipitate when working

up the pH 5 fraction, and for this reason was chosen for most experimental situations involving lysine. Table IV also contains data which demonstrate that potassium ion worked better for stimulation of polyphenylalanine incorporation. When potassium buffers were used to gain maximum incorporation, the pH 5 fraction was precipitated with buffer pH 5N, but washed with buffer pH 5'K to insure removal of contaminating ammonium ions.

Optimum ribosome concentration. At the start of this investigation, a ribosomal concentration had to be chosen. Since the frozen ribosomal stock solutions were usually made up to 25 mg./ml., it was convenient to use 20 µl. of this solution for each incorporation tube. Since most experiments were done on a 12-trial scale, the aliquot of frozen ribosomal stock solution was 250 µl. This meant that optimum conditions were being sought for 500 µg. of ribosomes.

The results of a ribosome titration using identical incorporation conditions is shown in Table V.

TABLE V. RIBOSOME TITRATION USING 3 µLITERS OF POLY A.

Reaction Conditions	Corrected Counts
10 µliters Ribosomes	75,231
20 µliters Ribosomes	86,135
30 µliters Ribosomes	112,434
40 µliters Ribosomes	107,571

The background (for 20 µliters of ribosomes) was 1,289.

The maximum stimulation for 3 µl. of poly A was attained at about 500 µg. of ribosomes per one milliliter incorporation.

This result was obtained independently by Kuriki (1968), as a result of a much more thourough study. Adding additional poly A increased the optimum somewhat, but there was no directly proportional effect.

The effect of tRNA. The addition of tRNA increases the efficiency of incorporation, as concluded from the data presented in Figure 4.

The effect of pH 5 solution. The choice of 100 µl. of pH 5 solution was an adequate compromise between incorporation efficiency and ingredient "cost", as the data of Figure 5 suggest.

The effect of poly A. Table VI records the results of an experiment in which poly A concentration was titrated against incorporation efficiency (using 500 µg. of ribosomes). The choice of 3 µl. (50 µg.) was shown to be reasonable, and internally consistent with the notion that the micro assay system was based to some extent on this ingredient.

TABLE VI. POLY A TITRATION USING 550 µGRAMS OF RIBOSOMES.

Reaction Conditions	Corrected Counts
3 µ liters Poly A	70,244
6 µ liters Poly A	72,243
12 µliters Poly A	75,932
24 µliters Poly A	59,724

The background (for 6 µ liters of poly A) was 839.

Discussion

The significance of the preparative techniques developed in this study may be summarized as follows: (1) the isolated

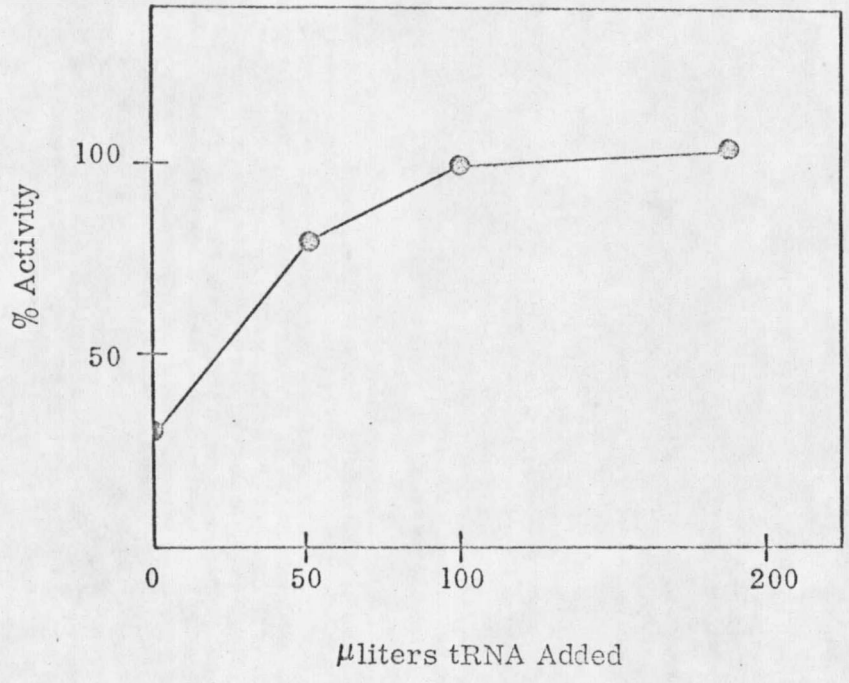


Figure 4. The effect of transfer RNA on incorporation efficiency. This titration was done using the normal incorporation system with 500 μg of ribosomes.

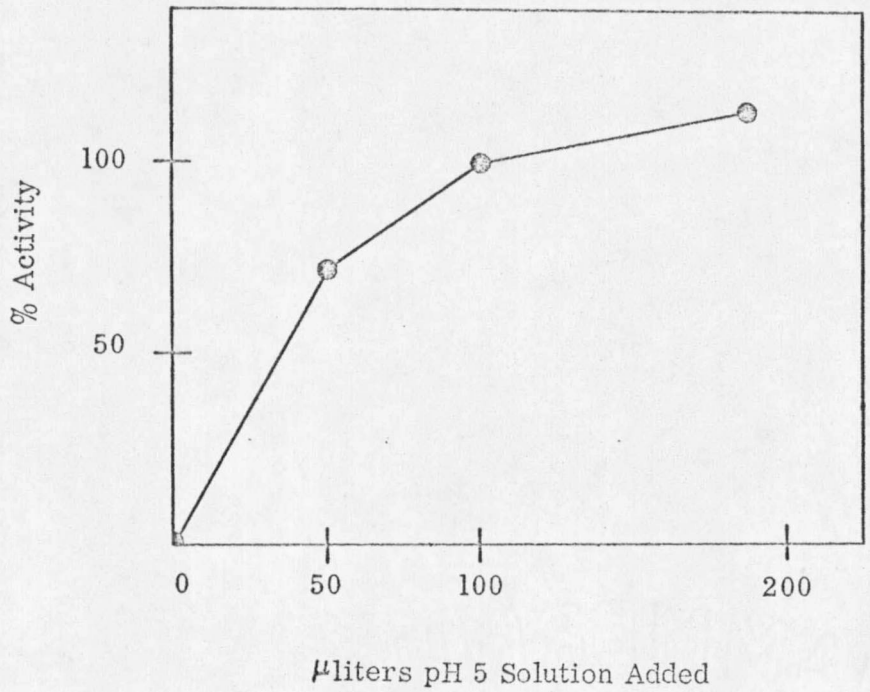


Figure 5. The effect of pH 5 solution on incorporation efficiency. This titration was done using the normal incorporation system with 520 μg of ribosomes.

ribosomes are pure; (2) the ribosomes are isolated in good yield; and (3) the isolation method is easy and reproducible.

The small-scale assay method developed in this study is economical and reproducible, but has the following limitations: (1) the pH 5 fraction (as prepared by the method reported in this study) may vary. As a result, day to day comparisons of experimental results are difficult, and variable testing must be carried out utilizing the same pH 5 preparation. This can lead to inconveniently large and wieldy experiments. (2) It should be emphasized that the use of synthetic messages for ribosomal mechanism of action studies is restricted to those functions which do not involve specialty codons. Therefore, the poly A- and poly U-directed assay system here developed has an uncertain applicability.

Nevertheless, the methods presented in this study are the methods of choice when initially devising a ribosomal in vitro assay system. The chromatographic method for the preparation of bacterial ribosomes in particular is superior to any preparative method known to the author.

Certain conclusions about the nature of the Escherichia coli ribosome appear possible upon consideration of its chromatographic behavior. On DEAE-cellulose, the properties of E. coli ribosomes are clearly analogous to those of normal RNA. This fact, taken together with dye-binding studies (Cotter, 1967), indicates that the exposed portion of the ribosome is RNA. This would place the ribosomal proteins either in the

interior of the ribosome -- a possibility which is incompatible with the reconstitution experiments (Traub, 1968), and optical rotatory dispersion experiments conducted on free rRNA and ribosomes (Sarkar, 1967) -- or together in a relatively small location on the ribosomal surface. This latter possibility is attractive in view of the "pocket" or "cleft" revealed by X-ray structural determinations to be the active site of many enzymes. This idea also fits with the protection experiments which showed that protection was afforded the photolabile group by prior mRNA attachment. Support for this cleft model has recently been provided by the publication of a study involving interpretations of electron micrographs (Bruskov, 1968).

APPENDIX 2

The effect of thiacyanines on ribosomes

Introduction

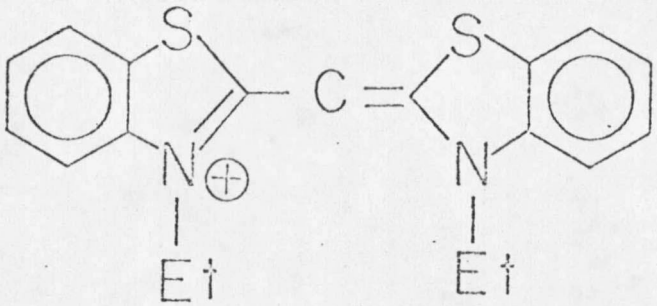
During the course of this investigation, selected compounds of the cyanine dye class were investigated as potential sensitizers for the photodynamic degredation of ribosomes. This idea was based on the fact that certain of the cyanines are used as photosensitizers in commercial photographic practice. None of the thiacyanines tested showed any promise as ribosomal photo-sensitizers. However, they all exhibited a very interesting dark reaction.

The effect of 3,3'-diethylthiacyanines

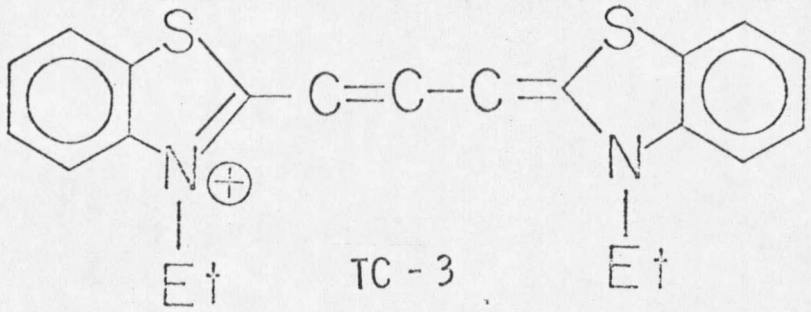
Figure 1 presents the structures of the three dyes used in the investigation. It can be seen that the carbon chain length between the benzothiazole rings has a marked effect upon light absorbtion in the visible region.

Table 1 displays data taken from experiments which illustrated the effect of these thiacyanines upon ribosomal incorporation of radioactive amino acids. All three cyanines had a pronounced dark reaction.

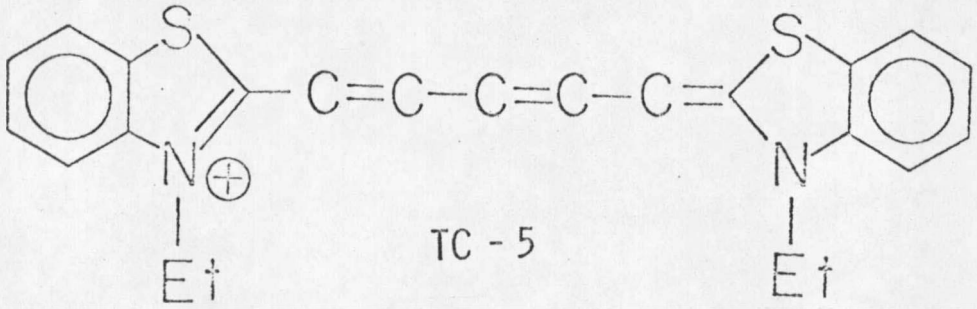
Attempts to demonstrate a photo-effect with these dyes led to the discovery that TC-3 and TC-5 were very rapidly bleached upon exposure to light in the presence of RNA (rRNA, tRNA, and especially poly A). This bleaching effect, and in the case of TC-3, the concomitant formation of a white precipitate, could be correlated with a slight reversibility of their dark effect. TC-1 did not bleach appreciably, and



TC - 1



TC - 3



TC - 5

Figure 1. Structures of three thiacyanines. Water : ethanol solutions (1 : 1 v/v) of these dyes were yellow (TC-1), magenta (TC-3), and blue (TC-5). The dyes were used as iodide salts.

TABLE I. THE EFFECT OF THIACYANINES ON RIBOSOMES.

Reaction Conditions	Corrected Counts	% Activity
Normal	1099	100
TC-3 in the dark	473	43
Experiment 1 with light	294	27
Experiment 2 with light	574	46
TC-1 in the dark	220	20
Experiment 2 with light	115	10
TC-5 in the dark	516	47
Experiment 2 with light	568	52

Dye solutions were made up to 0.2 μmoles with water : ethanol (1 : 1 v/v). The normal incorporation contained this same amount of ethanol. Experiment 1 had buffer + dye + poly A, then light, followed by the addition of everything else except pH 5. Experiment 2 had buffer + dye + tRNA + ribosomes + ATP, CTP, GTP, PEP, pyruvate kinase, then light, followed by the addition of everything else except pH 5. Both experiments were initiated by the addition of pH 5. The background was 67. Light duration was 60 seconds.

showed an increase in its inhibitory effect upon exposure to light.

Discussion

It should be pointed out that this effect of the thiacyanines is better (on the basis of concentration) than most ribosomal antibiotics. Since preparative methods are available which allow the synthesis of practically any thiacyanine, it would appear that these dyes constitute a unique tool with

which to probe the chemical structure / function relationship between the ribosome and an inhibitor of a ribosomal function.

Furthermore, it should be possible to synthesize thiacyanines with constituents which would allow penetration of the bacterial cell wall (or mammalian cell membrane), but which would not interfere with the inhibitory effect of specific dyes. Compounds of this type suggest the creation of tailor-made antibiotics by known synthetic methods -- certainly a unique approach to the study of antibiotics, a subject which has heretofor been limited to varying the structure of natural antibiotics isolated from living organisms.

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