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THE EARLY STEPS IN PROTEIN SYNTHESIS AND THEIR REGULATION:  
A BACKGROUND STUDY RELATED TO THE BIOLOGICAL EFFECTS OF RADIATION

Progress Report

for Period July 1, 1975 - June 30, 1976

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ANNUAL REPORT

UNITED STATES ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION

CONTRACT NO. AT(11-1)-2403 (Formerly AT(30-1)-2643)

July 1, 1975 - June 30, 1976

THE EARLY STEPS IN PROTEIN SYNTHESIS AND THEIR REGULATION:

A BACKGROUND STUDY RELATED TO THE BIOLOGICAL EFFECTS OF RADIATION

Abstract

This is a continuing study of protein synthesis, involving 1) a search for the role of Ap<sub>4</sub>A and other unusual nucleotides in growth regulation; 2) studies of the mechanism of action of aminoacyl-tRNA ligases and the effect thereof on protein synthesis; 3) a search for new regulators of the translation step, in cell-free systems; and 4) an effort to improve the sensitivity and quantitation of chemical sequencing at the 3'-end of messenger RNA.

Ap<sub>4</sub>A has been consistently found in *in vivo* studies of the rat, in ascites cells in suspension, and in human hepatoma cells in tissue culture, in 10<sup>-6</sup>-10<sup>-7</sup> molar concentrations. Its concentration diminishes with anoxia. Mutant aminoacyl-tRNA ligases have been found with greatly diminished enzymatic properties and structural differences from their normal counterparts. Inhibition studies of purified methionyl-tRNA synthetase have been aimed at the site of action with its cognate tRNA. Conditions for optimizing cell-free translation of 35S AMV RNA in wheat germ and reticulocyte systems are being worked out. The sensitivity of chemical sequencing has been improved, down to the subnanomolar level.

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\*Paul C. Zamecnik, M.D. - Principal Investigator - Percentage of Effort: 25%

## I. Scope of Project - Introduction

This contract has for many years been the principal support for our work on protein synthesis. It enabled us to find the first cell-free system for investigating this obscure area of biochemistry, thereby identifying a) the aminoacyl-tRNA ligases as the initial catalysts in activation of amino acids in an interaction with ATP; b) the ribosomes (microsomes at that time) as the site at which the polypeptide synthesis occurred; c) transfer RNA as an obligatory intermediate between the reactions of a) and b); d) GTP and polypeptide polymerase as participants in the chain lengthening steps; and e) Ap<sub>4</sub>A as a potential regulator nucleotide. After our initial establishment of a cell-free system in rat liver, we subsequently developed the first cell-free protein synthesizing systems in higher plants and in bacteria.

Our studies with Ap<sub>4</sub>A and related compounds are becoming increasingly interesting. Small technical improvements have made investigation of the possible role of Ap<sub>4</sub>A in metabolic regulation more feasible. We have also recently reported the formation of a new compound, or really two closely related compounds, related to the back reaction of the aminoacylation step in protein synthesis. In the presence of lysyl-tRNA ligase, ATP, lysine, and the so-called magic spots I and II (ppGpp and pppGpp), the compounds ApppGpp and AppppGpp are synthesized in vitro. The 5'-diphosphorylated end of a high molecular weight RNA is under the same circumstances converted to ApppX-RNA, a new type of "capping" reaction.

Following the discovery of messenger RNA, due particularly to Nirenberg and Matthaei, it became possible to add viral mRNA to cell-free systems and to look for viral specific proteins. Thus a multiplicity of factors related to the initiation, propagation, termination, and species specificity of the nascent peptide chains became recognized. These were not appreciated nor necessary when the simplified homopolymers of RNA used by Nirenberg, Ochoa, and others were first employed. We have taken up this pursuit, and use the Avian Myeloblastosis Viral 35S RNA messenger in the search for factors not yet clearly defined in protein synthesis. In regard to an earlier step in protein synthesis, we are conscious of how important and resistant to clarification the interaction between an aminoacyl-tRNA ligase and its cognate synthetase is. Herein lies a code, separate from codon-anticodon association of nucleotides, which determines the specificity of the primary sequence of a protein being synthesized. The specificity must depend on interaction of certain nucleotide bases of a transfer RNA with amino acid side chains of its cognate synthetase. One recent approach of ours has been to produce mutations of an aminoacyl-tRNA ligase, and to try to analyze the effect on the interaction with its cognate tRNA. A second approach has been to study the effect of site specific reagents on the properties of purified methionyl-tRNA synthetase.

The effort to improve chemical sequencing of high molecular weight RNA has a bearing on the mechanisms of translation and their regulation. It is known that a 3'-terminal poly A segment is a feature of a predominant number of messenger RNAs. Just internal to the poly A segment in the Rous sarcoma virus transforming and non-transforming 35S RNAs is a segment of oligonucleotides which is common to both. The function of this oligonucleotide segment, presumably present also in AMV 35S RNA, is unknown but is thought to play some regulatory role in the translation function of the viral RNA. Our 3'-end sequencing technique, when sufficiently sensitive to deal with the minute amounts of 35S RNA available from oncogenic viral sources (and AMV provides by far the largest amount), will essay to sequence this interesting oligonucleotide segment just internal to the poly A terminus.

Thus our findings during the past year have been built on earlier work here and elsewhere in the expanding field of cell-free protein synthesis; and they are poised at uncovering factors or mechanisms involved in the regulation of protein synthesis. We are devoting increasing attention to the translation step as well as to the amino acid activation step. In next year's proposal the words "early step" in protein synthesis are therefore omitted from our title.

The investigations described herein are a result of a consciously interrelated effort to gain insight into factors involved in the biosynthesis of proteins and the regulation of growth. Although not all investigations mentioned receive renumeration directly from the current contract the ERDA (AEC) has been for many years the major source of this interrelated effort (see Proposal).

We are conscious of the fact that there are areas of overlap in the proposal and this annual report. Parts of the latter seem better presented with our plans for the future rather than as isolated reporting of work accomplished. In the interest of completeness they are included in both and we are aware that the same wording may appear in both places. In addition, the proposal is referenced more thoroughly than this report of work accomplished during the past year.

In this introduction we have outlined the scope of the projects and have indicated how they interdigitate with our overall program of research. The reports are presented here as separate units for clarity but there are interrelationships between the projects and the viral RNA samples are shared by all of us.

## II. Specific Reports

### A. The In Vitro and In Vivo Utilization of Aminoacyl Adenylylates as Intermediates for Adenylylation Reactions - E. Rapaport & P. Zamecnik

The first step in protein synthesis which was discovered some 20 years ago in these laboratories (1,2) includes the formation of the aminoacyl adenylylate. While its role as an obligatory intermediate in the aminoacylation reaction was recently challenged (3,4) it has received support difficult to refute; during the past year an aminoacyl adenylylate has also been shown to participate in the activation reaction of a well-known carcinogen (5), due to its reactivity as a mixed anhydride of carboxylic and phosphoric acids. We have now shown it to be a potentially important reactive adenylylation intermediate in several in vitro and in vivo systems. ppGpp (MSI) reacts rapidly with aminoacyl adenylylates in an in vitro system to yield almost exclusively A(5')ppp(5')Gpp (6). This reaction may constitute a step in the metabolism of ppGpp, which is known to undergo rapid turnover (7,8). It may also shed some light on the unexpected reversal of the stringent response in amino acid starved bacteria by chloramphenicol (7) and other inhibitors of protein synthesis (9), which affect initiation or elongation but not the amino acid activating process, thus leading to accumulation of aminoacyl adenylylates. Hydroxylamine, which reacts with aminoacyl adenylylates (10) has been shown by us and others (7) to lead to the accumulation of ppGpp in bacteria.

In addition to ppGpp, ATP (pppA) has been known to react in the back reaction of the amino acid activation process (Figure 1b, c).

- a.  $\text{pppA} + \text{aa}_1 + \text{Eaa}_1 \rightleftharpoons \text{aa}_1-\text{pA}\text{--Eaa}_1 + \text{pp}$
- b.  $\text{aa}_1-\text{pA}\text{--Eaa}_1 + \text{ppGpp} \rightleftharpoons \text{A}(5')\text{ppp}(5')\text{Gpp} + \text{aa}_1 + \text{Eaa}_1$
- c.  $\text{aa}_1-\text{pA}\text{--Eaa}_1 + \text{pppA} \rightleftharpoons \text{A}(5')\text{pppp}(5')\text{A} + \text{aa}_1 + \text{Eaa}_1$

Figure 1

The participation of ATP in the back reaction of the amino acid activation yielding Ap<sub>4</sub>A was discovered some 10 years ago (11-14) in these laboratories and a specific enzyme which cleaves Ap<sub>4</sub>A to AMP and ATP has recently been isolated by another group from rat liver (15). We have recently developed a sensitive assay for Ap<sub>4</sub>A in a human hepatoma cell line, and in in vivo normal or regenerating rat liver, which greatly facilitates exploration of our previous finding of this compound in *E. coli*, ascites cells, and rat liver (13,14). Using high specific activity [<sup>3</sup>H]adenosine or <sup>32</sup>Pi we have positively identified labeled Ap<sub>4</sub>A in concentrations ranging from 10<sup>-6</sup> to 10<sup>-7</sup>M in these two model systems. The assay procedure includes chromatography of acid soluble nucleotides on a DEAE Sephadex -7M urea column, which separates nucleotides according to their negative charge, followed by chromatography of appropriate fractions on a DEAE cellulose column, which separates small amounts of Ap<sub>4</sub>A from ATP due to its extra retardation in this system. The molar concentration of Ap<sub>4</sub>A is determined based on the ATP concentration, which is known to be approximately 3.0 x 10<sup>-3</sup>M in these systems (16). The ATP to ADP ratio, which is also provided by each assay, is indicative of whether anoxic conditions, which tend to lower the ATP level, have been created during the anesthesia, liver fixation and work-up, as well as in tissue culture manipulations. We have also previously determined ATP concentrations directly (Stephenson, M.L. and Zamecnik, P.C., unpublished), using the chemical luminescence-firefly luciferin technique.

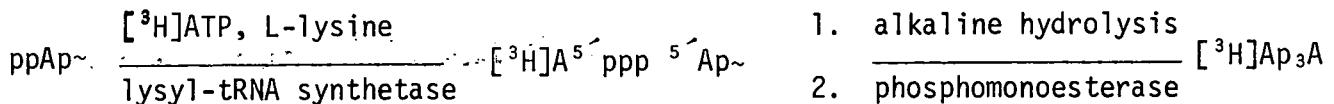
We have recently succeeded in applying our new post-isolation  $^{32}\text{P}$ -labeling technique to the 5'-end group analysis of 35S AMV RNA (17). The reaction utilizes the diimidazolidate of  $[^{32}\text{P}]$ orthophosphate, which reacts exclusively with primary phosphates. It reacts with phosphorylated 5'-ends while 5'-OH ends are not affected. The  $^{32}\text{P}$ -labeled 35S AMV RNA was isolated without apparent degradation on dimethyl sulfoxide-sucrose-rate-zonal gradients. Alkaline



hydrolysis,  $\text{T}_1$ -ribonuclease and pancreatic ribonuclease digestion revealed a population of 10-20 percent of diphosphorylated species before the labeling reaction. The use of  $[\gamma-^{32}\text{P}]$ ATP-polynucleotide kinase labeling procedure, with and without prior use of alkaline phosphatase, indicated that a sizeable fraction of 5'-ends of the 30-35S AMV RNA are not phosphorylated. A 5'-terminal group of 35S AMV RNA was identified as  $\text{HOApGp}$  with a small population of ppApGp. The polynucleotide kinase labeling reaction was equally effective when carried on the 35S or 70S AMV RNA, thus indicating that the 5'-ends of the 35S RNAs in the 70S RNA complex are well exposed.

An enzymatic process which has been known to react with di- and triphosphorylated nucleotides was applied to the post-isolation labeling of a high molecular weight RNA species with a diphosphorylated 5'-end.

When the 70S AMV RNA was reacted with  $[^3\text{H}]$ ATP, L-lysine and purified lysyl-tRNA synthetase, which was completely free of nuclease activity, the  $^3\text{H}$ -(adenosine-5') triphosphate derivative of the 70S AMV RNA was produced and isolated. Alkaline hydrolysis of the 5'-end-modified and otherwise unaltered 70S RNA yielded  $[^3\text{H}]Ap_3Ap$  which was converted to the known  $Ap_3A$  by alkaline phosphomonoesterase. The "capping" of a diphosphorylated or triphosphorylated species by an active intermediate such as aminoacyl adenylate may even be of a more general nature.



It is possible that a considerable fraction of the 30-35S RNA molecules may carry the "G-7me-pppG-2'ome" terminus of Shatkin and others and this possibility remains to be answered. Our experiments only state that not all 5'-ends of high molecular weight RNA molecules, as extracted by us from infected chick plasma, bear this frequently found capping constellation.

References

1. Hoagland, M.B. (1955) *Biochim. Biophys. Acta* 16, 288-289.
2. Hoagland, M.B., Keller, E.B., and Zamecnik, P.C. (1956) *J. Biol. Chem.* 218, 345-358.
3. Loftfield, R.B. (1972) in *Progress in Nucleic Acid Research and Molecular Biology*, Vol. 12, pp. 87-128.
4. Lovgren, T.N., Heinonen, J., and Loftfield, R.B. (1975) *J. Biol. Chem.* 250, 3854-3860.
5. Tada, M. and Tada, M. (1975) *Nature* 255, 510-512.
6. Rapaport, E., Svhovec, S.K., and Zamecnik, P.C. (1975) *Proc. Nat. Acad. Sci. USA* 72, 2653-2657.
7. Lund, E. and Kjelgaard, N.O. (1972) *Eur. J. Biochem.* 28, 316-326.
8. Fiil, N., von Meyenburg, K., and Friesen, J.D. (1972) *J. Mol. Biol.* 71, 769-783.
9. Hershko, A., Mamont, P., Shields, R., and Tomkins, G.M. (1971) *Nature New Biology* 232, 206-211.
10. Jencks, W.P. and Lipman, F. (1957) *J. Biol. Chem.* 225, 207-223.
11. Zamecnik, P.C., Stephenson, M.L., Janeway, C.M., and Randerath, K. (1966) *Biochem. Biophys. Res. Comm.* 24, 91-97.
12. Randerath, K., Janeway, C.M., Stephenson, M.L., and Zamecnik, P.C. (1966) *Biochem. Biophys. Res. Comm.* 24, 98-105.
13. Zamecnik, P.C. and Stephenson, M.L. (1969) in *Alfred Benzon Symposium I: The Role of Nucleotides for the Function and Conformation of Enzymes*, eds. Kalckar, H.M., Klenow, H., Munch-Petersen, G., Ottesen, M., and Thuysen, J.M. (Munksgaard, Copenhagen), pp. 276-291.
14. Zamecnik, P.C. and Stephenson, M.L. (1968) in *Regulatory Mechanisms for Protein Synthesis in Mammalian Cells*, eds. San Pietro, A., Lamborg, M.R., and Kenney, F.T. (Academic Press, Inc., New York), pp. 3-16.
15. Lobaton, C.D., Vallejo, C.G., Sillero, A., and Sillero, M.A.G. (1975) *Eur. J. Biochem.* 50, 495-501.
16. Bucher, N.L.R. and Swaffield, M.N. (1966) *Biochim. Biophys. Acta* 129, 445-459.
17. Rapaport, E. and Zamecnik, P.C. (1975) *Proc. Nat. Acad. Sci. USA* 72, 314-317.

B. Studies on a Cell-Free System, Dependent Upon Added Viral mRNA for Translation, and Sensitive to the Addition or Withdrawal of "Factors" for Optimal Translation - M. L. Stephenson

1. Introduction, Objectives, and Findings to Date
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1. Introduction, Objectives, and Findings to Date

Our long-standing studies under predecessors of this Contract have been on the basic mechanisms involved in the biosynthesis of proteins. These have been extended from the use of normal tissues to include an investigation of what happens when oncogenic RNA viruses subvert this normal machinery. In this event the cell is not killed, but part of its protein synthesizing machinery is "turned-on" to produce viral proteins. Such studies may help our understanding of normal growth regulation as well as some of the factors involved when the orderly progression of events is disrupted.

Cell-free protein synthesizing systems are being investigated, particularly the requirements for specific nucleic acid and protein factors. In long-range plans special attention will be devoted to attempts to identify the production of the viral RNA directed DNA polymerase (reverse transcriptase) in such a cell-free system. A study of its mechanism of synthesis may help in understanding of the process of viral oncogenesis and in addition its detection by enzyme amplification may provide us with a biochemical "handle" with which we may investigate factors necessary for production of a biologically active protein in a cell-free system.

In order to approach this long-range goal it is first necessary to establish a reproducible cell-free incorporating system which responds to the addition of viral mRNA to produce viral specific proteins. For this reason the bulk of our studies have been concerned with our investigation of various cell-free systems and our plans to elicit a more reproducible and larger response to the added viral RNA than has heretofore been obtained by us and others. We already have evidence in the wheat embryo and the reticulocyte systems of a nice response to the added RNA and in the wheat embryo we find viral specific proteins in the correct size range for the viral RNA dependent DNA polymerase. Similar experiments are in progress using a purified reticulocyte system.

We have calculated from the methionine incorporated into protein bands that it is reasonable to look for the viral enzyme using enzymatic amplification both as a tool to increase its limits of detection and to establish the fact that the viral product of the cell-free system has biological activity.

## 2. Work Accomplished

### a. Introduction

For the most part we have studied the response of the wheat embryo system to AMV heated 70S RNA. In conjunction with these studies we have also used the rabbit reticulocyte cell-free system consisting of clean ribosomes plus factors. Both types of experiments are reported below. In order to use any of the systems it has been critical to have good viral RNA samples, and some effort has been expended in the isolation of intact 70 or 35S RNA. The following findings have also been discussed in our proposal along with much more complete reference to the work of others. Our primary concern is to establish a reproducible protein synthesizing system with low endogenous incorporation and a high viral specific response to added viral RNA.

### b. Findings from our investigations of the wheat embryo system

#### (1) Incubation conditions

For the most part we have used a wheat embryo system similar to that of Roberts and Paterson (1), using an S-30 fraction (sometimes pre-incubated) isolated from Sephadex G-25. Our optimal conditions of KCl (40 mM), Mg(Ac)<sub>2</sub> (1.5 mM), pH 8.5, spermine (80 mM), amino acids (.030 mM each), wheat embryo extract, time, temperature, and avian myeloblastosis viral heated 70S RNA (8-12  $\mu$ g/50  $\mu$ l) have been elaborated for labeling of alkali-stable, TCA precipitable material using <sup>35</sup>S-methionine or <sup>3</sup>H-leucine. We normally achieve a three- to five-fold stimulation on the addition of heated 70S AMV RNA to a pre-incubated Sephadex-treated wheat embryo system under these conditions, a stimulation greater than that published recently from other laboratories, but still far from what one would hope for optimally.

It is well known that optimal conditions for one type of mRNA are not necessarily the same as for others. For example, globin RNA has a much higher KCL optimum than AMV RNA in the wheat embryo system. Some of the conditions are optimal over a very narrow range, for example, Mg++ and KCL concentrations. One surprising finding is the necessity for a higher pH (pH 8.5) than published values, which are about pH 7.4 in all cell-free systems studied. There is a need to determine the factors responsible for such enormous variations in response to the added mRNAs, and our very recent findings with the reticulocytes in conjunction with our wheat embryo findings should help us to pinpoint areas of translational control, i.e., ribosomal binding, initiation, elongation, and release of newly formed viral peptides. We have made several attempts to stimulate the incorporation with other fractions from the wheat embryo extract (for example, "initiation factors" from salt washed ribosomes) and from the host myeloblast, with as yet inconclusive results.

#### (2) Investigations of proteins synthesized in response to added viral RNA

The 35S methionine labeled extracts have been applied to 1.5 mm thick slab gels of polyacrylamide containing SDS (2,3). The gels were dried following electrophoresis and radioautographs were made. The sensitivity of detection has been substantially increased by the addition of a scintillator to the gels (4,5). The results indicate that the bulk of the counts obtained upon stimulation by the viral RNA are different from the endogenous protein synthesis (see Figure 1, which is also in our proposal). Compare band A with B. These are radioautographs of incubation mixtures applied directly to the gels. A is the control and sample B has heated 70S RNA added.

Many protein bands seen in the radioautographs formed in response to viral RNA do not coincide with the stained wheat embryo proteins, while some others are questionable. Immune precipitation of the viral antigen proteins using pig anti-AMV reveals many bands of radioactivity in the precipitate from the incubation containing AMV RNA (see Figure 1D). The antibody was made from detergent disrupted total virus. Little radioactivity can be detected on the gels of the control samples (see Figure 1, A and C); viral related proteins were found only when the viral RNA was present. At the present moment we can conclude that we are observing proteins made in response to AMV RNA and many but not all of them appear to be antigenically related to AMV (see Figure 1D). Some but very few appear to correspond to known viral proteins. The stained gels are not shown here. We have not yet identified the bands corresponding to the viral polymerase in these experiments. Further experiments to pinpoint the viral specific proteins using more specific viral antibodies are in progress (i.e. by using antibodies to GS antigens). We have continued to improve our immunoprecipitation techniques (6-8) and are currently using antibody made against one specific viral protein (P-27). Precursor viral proteins or hydrolyzed viral proteins may also react with the antibodies made against total viral proteins. Because of this fact some of our labeled bands may be either precursors of or breakdown products of the actual viral proteins unless they coincide exactly with bands of known viral proteins.

Since most of the labeled bands from the immune precipitate do not coincide with viral proteins, we have planned to follow the order of labeling of proteins by the use of a short incubation of high specific activity  $S^{35}$  methionine followed by unlabeled methionine. The precursor protein relationship for the *in vivo* formation of AMV proteins has been studied by Eisenman (9) and Naso et al. (10) and certain sizes of precursors are known. These are not seen in the intact virion. In cell-free systems, these relationships are less certain, and are under current investigation. The profiles from slab gels of each time point should indicate whether the proteins made in response to AMV RNA are increasing in size as the incubation progresses. One experiment has indicated that this is the case but that there is little subsequent cleavage in the wheat embryo system. Figure 1B has indicated that by 30 minutes (with no chase) all sizes of proteins are labeled in response to the viral RNA.

### c. Reticulocyte cell-free system

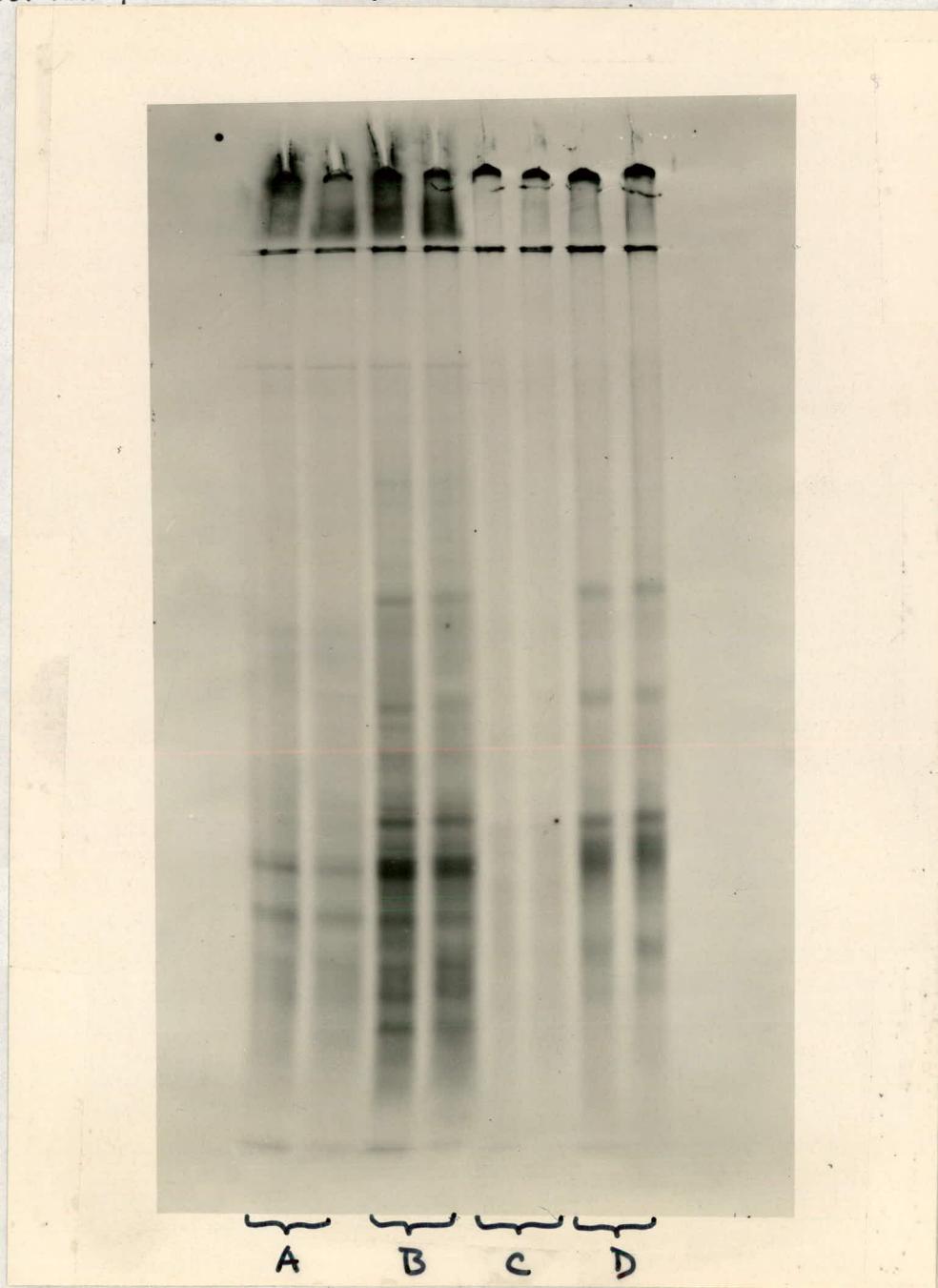
Current experiments carried out with Dr. J. Gilbert using purified rabbit reticulocyte ribosomes plus factors indicate a very large stimulation upon addition of the viral RNA. Experiments are in progress to identify these proteins. Bloemendal et al. (11,12) have recently used a reticulocyte lysate and indicated that although there is little or no overall stimulation by AMV RNA, in contrast to our 20-fold stimulation, some viral specific proteins can be detected following antibody precipitation. Very recent experiments indicate that some of the stimulation due to AMV RNA in our ribonuclease treated reticulocyte system is due to the production of reticulocyte proteins and only some are viral specific.

One of the most serious problems in almost all systems is that of obtaining ribosomes and factors free of endogenous mRNA. There have been several reports that even though washed, ribonuclease treated reticulocyte ribosomes have almost no incorporation, the addition of exogenous globin mRNA in some fashion causes the fragments of remaining endogenous RNA to become active and globin specific for the host mRNA is synthesized (J. Last, personal communication). We too are observing this effect in the reticulocyte system in response to added AMV RNA. It is essential to run gels to see what proteins are being formed in response to the exogenous RNA. Stimulation of protein synthesis by added mRNA over the control system means little unless the new proteins can be identified as being host or viral specific.

## LEGEND TO FIGURE

Figure 1 - Radioautograph of 14 percent polyacrylamide, 0.1 percent SDS slab gel containing samples of  $S^{35}$  labeled wheat embryo extracts both before and after precipitation of the proteins with anti-AMV serum.

- A - duplicate control extract
- B - duplicate control extract plus  $\Delta 70S$  AMV RNA
- C - Anti-AMV pellet from aliquot of control
- D - Anti-AMV pellet from sample B containing  $\Delta 70S$  AMV RNA



Our evidence using the wheat embryo system suggests that viral specific precursor proteins are made, but we do not find many viral proteins. This agrees with Sussman et al., who indicate that this system does not contain the specific cleavage enzymes necessary to form growth hormone from pro-growth hormone (13). On the other hand, Salden et al. (11) using the reticulocyte lysate system indicated the addition of AMV RNA resulted in precursor viral protein synthesis but in addition in this system there may have been some cleavage of the precursor proteins to virus specific proteins. Since we find some viral specific proteins produced in our current reticulocyte experiments a combination of each should enable us to isolate and study the cleavage enzymes (i.e., addition of reticulocyte factors to wheat embryo ribosomes).

We are indebted to Dr. Jack Gruber of the Office of Program Resources and Logistics of the Virus Cancer Program for supplies of avian myeloblastosis virus, generously furnished by Dr. and Mrs. Joseph Beard and for antisera to total AMV proteins and AMV P-27 obtained from Dr. Roger Wilsnak.

#### d. Studies with viral RNA

We have shown earlier that the 35S RNA of AMV terminates in adenosine at the 3'-end and that all the poly(A) is at that end of the molecule (14,15). We prepared some 35S RNA from heated 70S AMV RNA for Drs. Ursula Heine et al. to examine in the electron microscope (16). In addition to the large RNA they find that there are statistically significant smaller size units in the peak samples of 35S RNA analyzed. The significance of these "subunits" is not clear at present (Figures 2, 3, and 4, taken from Heine et al. (16)).

There is little difference whether AMV heated 70S RNA or 35S RNA is used in this wheat embryo system. Unheated 70S RNA is less efficient but not markedly so. Although the stimulation to date is not remarkable, it is reproducible and we have started to use it to investigate various RNA fractions.

When 70S RNA was heated to obtain the 35S RNA following sucrose gradient fractionation, a pooled RNA fraction sedimenting between about 8S and 20S gave as much stimulation on a molar basis as that from the 35S RNA pooled peak. There are several interpretations possible for this situation, and we shall refrain from making a preference until polyacrylamide gel autoradiograph of viral specific proteins being synthesized have been made. Similar studies are currently being carried out with Rauscher virus by Gielkens et al. (17,18). Experiments are in progress to isolate the mRNA fraction of AMV using oligo(dT) cellulose and to try to pinpoint the active fraction which is stimulating the incorporation using gradients to isolate specific RNAs. Further knowledge of the initiation site or sites on the mRNA and the role of 5'-terminal "capping" is essential, and our studies on the RNA are aimed at answering some of those questions. These proposed experiments with AMV RNA, supported under our Virus Contract, are being carried out in conjunction with chemical investigation of the primary structure of the RNA. The oligonucleotide sequencing methods being perfected by Dr. Schwartz (see section E) should interdigitate with our efforts to gain an understanding of the functional capacity of this viral message, which plays a central role in the production of viral specific proteins when normal cells become infected with virus.

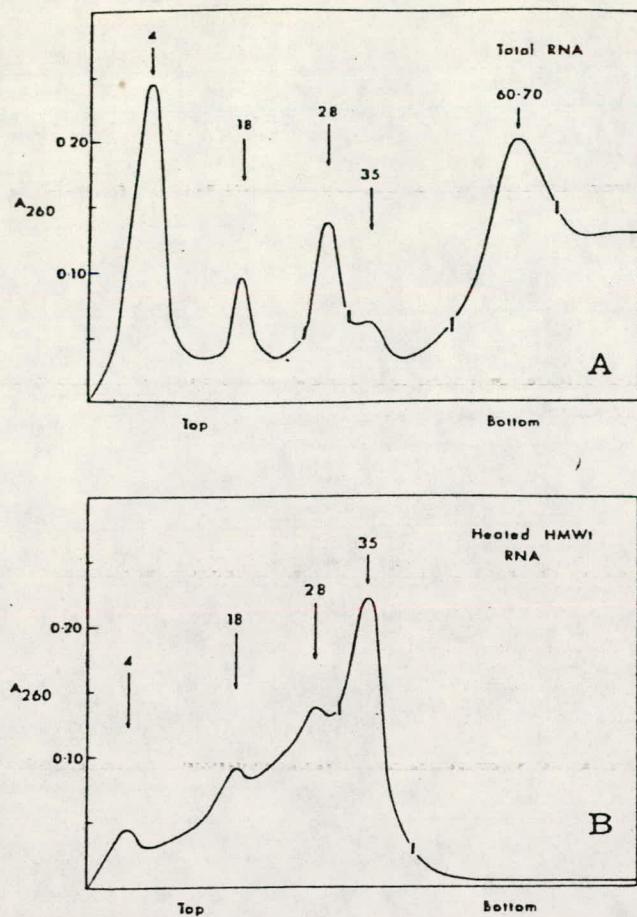


FIG. 2. (A) Sucrose gradient sedimentation of total RNA prepared from avian myeloblastosis virus (AMV). Linear sucrose gradients (5–30%) in high salt buffer (0.1 M LiCl; 0.05 M Tris, pH 7.4; 0.001 M EDTA; 0.2% Sarkosyl) were centrifuged at 40,000 rpm for 2½ hr at 5° in a Spinco SW 41 rotor. Peak fractions at 18 and 28 S were collected and the RNA was spread by the urea-formamide method to visualize single molecules. The 60–70S RNA was pooled as indicated by the vertical bars for isolation of 35S RNA.

(B) Sucrose gradient sedimentation of heated high-molecular-weight (HMWt) AMV RNA. 60–70S RNA of Fig. 1A was heat treated (73°, 3 min) and applied to a 10–30% linear sucrose gradient in low salt buffer (0.01 M LiCl; 0.05 M Tris, pH 7.4; 0.001 M EDTA). Centrifugation was for 5 hr at 40,000 rpm at 5° in a Spinco SW 41 rotor. Heat dissociation reveals the presence of smaller components (4, 18, and 28 S) in addition to the 35S viral RNA. The peak fraction at 35 S as indicated, was used for spreading.

Figures Taken from our Paper  
Heine *et al* (16)

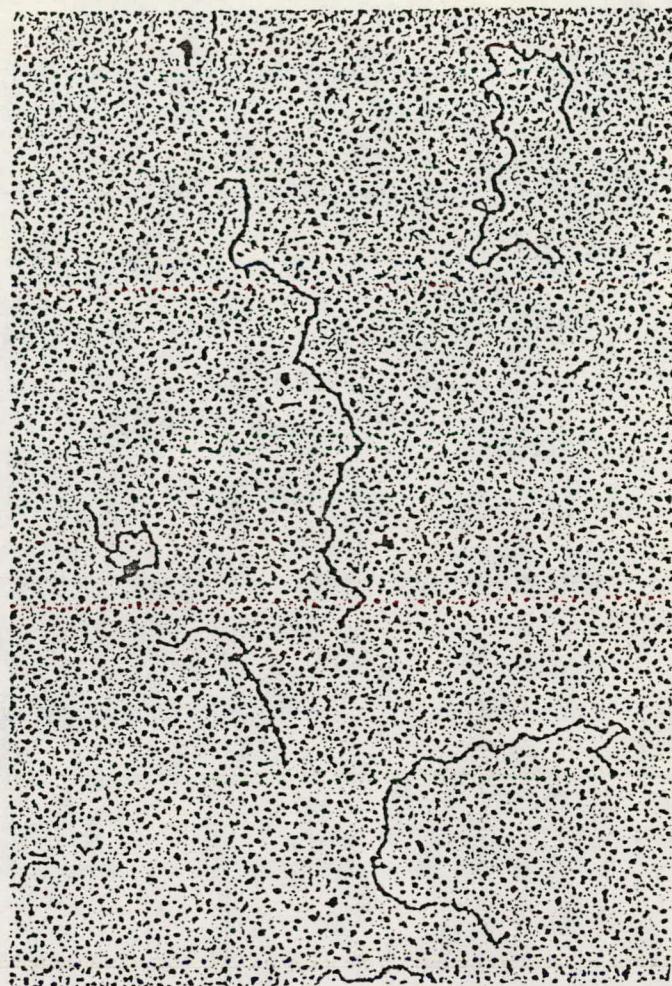


FIG. 3. AMV RNA molecules representing the 35S subunits obtained after heat dissociation of the 60–70S viral RNA. The longest molecules are about 3.0  $\mu$ m long. Shorter molecules are numerous. Magnification  $\times 38,000$ .

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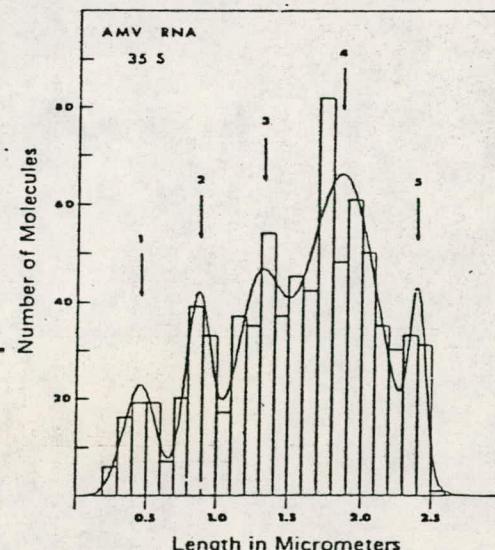


FIG. 4. Histogram of all subunits present in a given sample of 30–40S RNA obtained from high-molecular-weight AMV RNA by heat dissociation. Five distinct molecule sizes are present (1–5), the largest molecules (group 5) representing the 35S RNA (Exp. 2 in Table 1).

### e. Discussion

The work accomplished up to this point represents our effort to establish a cell-free system capable of making significant amounts of viral proteins. Without this we cannot ascertain the specific factors necessary to stimulate (or inhibit) their production.

We are specifically interested in detecting reverse transcriptase activity which should give us good evidence of the production of a biologically active viral specific protein in response to the added viral RNA. The viral protein might also be identified by other than enzymatic means such as immunoprecipitation against antibody directed against the purified DNA polymerase or by radioimmune assay (19), but these assays would not guarantee that the transcriptase has biological activity. Identification of a viral protein by other than immune precipitation techniques would eliminate the uneasiness some immunologists have about positive identification of known viral proteins by antibody precipitation. What is most important is the fact that we already have evidence that the wheat embryo and reticulocyte systems we are using are capable of making viral specific proteins in the molecular weight range of the reverse transcriptase in response to AMV-RNA. At present we don't know what extent of enzyme amplification we can expect but have calculated that it is reasonable to initiate such a project.

These long-range studies may provide some insight into the means by which RNA viruses alter normal cell protein synthesizing systems and in doing so may in turn help our understanding of the factors influencing the normal growth regulation processes. The studies are a logical extension of our years of basic work on normal systems.

## 3. References

1. Roberts, B.E. and Paterson, B.M. Proc. Nat. Acad. Sci. USA 70:2330, 1973.
2. Davis, B.J. Ann. N. Y. Acad. Sci. 121:404, 1964.
3. Reid, M.S. and Bieleski, R.L. Anal. Biochem. 22:374, 1968.
4. Bonner, W.M. and Laskey, R.A. Eur. J. Biochem. 46:83, 1974.
5. Laskey, R.A. and Mills, A.D. Eur. J. Biochem. 56:335, 1975.
6. Bolognesi, D.P., Ishizaki, R., Huper, G., Vanaman, T.C., and Smith, R.E. Virology 64:349, 1975.
7. Strand, M. and August, J.T. J. Virology 15:1332, 1975.
8. Fletcher, P., Nowinski, R.C., Tress, E., and Fleissner, E. Virology 64:358, 1975.
9. Vogt, V.M., Eisenman, R., and Diggelmann, H. J. Mol. Biol. 96:471, 1975.
10. Naso, R.B., Arcemond, L.J., and Arlinghaus, R.B. Cell 4:31, 1975.
11. Salden, M.H.L. and Bloemendaal, H. Biochem. Biophys. Res. Comm. 68:249, 1976.
12. Salden, M., Asselbergs, F., and Bloemendaal, H. Nature 259:696, 1976.
13. Sussman, P.M., Tushinski, R.J., and Bancroft, F.C. Proc. Nat. Acad. Sci. USA 73:29, 1976.
14. Stephenson, M.L., Wirthlin, L.R.S., Scott, J.F., and Zamecnik, P.C. Proc. Nat. Acad. Sci. USA 69:1176, 1972.
15. Stephenson, M.L., Scott, J.F., and Zamecnik, P.C. Biochem. Biophys. Res. Comm. 55: 8, 1973.
16. Heine, U.I., Weber, G.H., Cottler-Fox, M., Layard, M.W., Stephenson, M.L., and Zamecnik, P.C. Proc. Nat. Acad. Sci. USA 72:3716, 1975.
17. Gielkens, A.L.J., Van Zaane, D., Bloemers, H.P.J., and Bloemendaal, H. Proc. Nat. Acad. Sci. USA 73:356, 1976.
18. Salden, M.H.L., Gielkens, L.J., and Bloemendaal, H. Biochim. Biophys. Acta 425:208, 1976.
19. Panet, A., Baltimore, D., and Hanafusa, T. J. Virology 16:146, 1975.

C. Studies on the Mechanism of Action of Methionyl-tRNA Synthetase of *E. coli* - D. L. Coleman

1. Inhibition by Dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide
  - a. Sites of reaction
  - b. Conformational effects
  - c. Substrate-binding studies
2. Evidence that Dithiothreitol Interferes with the Binding of Methionyladenylate to MRS
3. References

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1. Inhibition by Dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide

We have been investigating methionyl-tRNA synthetase (MRS) of *E. coli* with the objective of gaining an understanding of the nature of the interactions whereby this enzyme can identify and form a complex with its cognate transfer RNA. One of the approaches which we have been using is a study of the effect of various site-specific reagents on the properties of the enzyme. Thus, previous work focused on the effect of p-hydroxymercuribenzoate on the enzyme (1).

A second reagent which we have investigated is dimethyl(2-hydroxy-5-nitrobenzyl) sulfonium bromide (DSB). As reported previously (2), methionyl-tRNA synthetase of *E. coli* is inhibited by DSB. The inhibition produced by DSB is irreversible, with the product of reaction of the reagent with groups on the protein being a covalently substituted derivative. Because there is a difference in the degree of inhibition as measured by the ATP-pyrophosphate exchange reaction from that observed with the aminoacylation reaction, we were interested in investigating the sites on the enzyme at which DSB reacts and in determining the effect of the reaction on other properties of the enzyme such as conformation and ability to bind substrates.

a. Sites of reaction

DSB has been found to react primarily with thiol and indole groups (3). Amino acid analysis of the native and modified enzymes has indicated that 14 + 7% of the tryptophan residues are modified in the course of the reaction, which is carried out at 22° for 15 minutes. On the basis of 27 tryptophan residues per enzyme molecule (4), we find that 4 + 2 tryptophan residues react with DSB. Similarly, amino acid analysis of the cysteine residues as cysteic acid (5) revealed that 4.2 + 1.4 out of a total of 24 per molecule are modified by DSB under the conditions used. Since the native enzyme is a dimer having one independent active site per monomer (6), these results have suggested that two tryptophan and two cysteine residues per monomer may react with DSB. Further, evidence has been reported that each monomer may have two homologous amino acid sequences arranged in a tandem sequence (7). Thus, there may be only two distinct reaction sites for DSB on the molecule, one being a specific tryptophan residue and the other a specific cysteine residue. The total number of DSB residues per protein molecule calculated on the basis of the amino acid analysis is 8.2 + 3.4. By a direct measurement of the 2-hydroxy-5-nitrobenzyl chromophore, we estimate that 5.6 moles of DSB react per mole of enzyme, a figure which is reasonably close to that obtained by amino acid analysis.

### b. Conformational effects

The effect of DSB modification on the conformation of MRS has been studied with the use of both fluorescence and circular dichroic spectroscopy. There is a significant loss in the observed intrinsic fluorescence of the modified enzyme as compared to the native enzyme. In order to determine how much of the loss in fluorescence is due directly to the modification of tryptophan residues and how much may be attributed to conformational perturbations, the fluorescence measurements were also carried out in the presence of 1 percent sodium dodecyl sulfate (SDS). Under these denaturing conditions, the fluorescence of the unmodified enzyme is reduced to 45 percent of that for the native enzyme while, in the case of the modified enzyme, the fluorescence is reduced to 84 percent of that observed in the absence of SDS. Thus we may conclude that more than half of the decrease in fluorescence observed for the undenatured modified enzyme is associated with conformational perturbations.

In the presence of SDS the ratio of the fluorescence of the modified enzyme to that for the unmodified enzyme should provide a measure of how many tryptophan residues have reacted with DSB. Such a calculation indicates that 39 percent of the tryptophan has been modified. This figure is significantly greater than the value obtained by amino acid analysis. Possible reasons for the difference in results obtained by the two methods are being investigated and include: (i) a decrease in the residual fluorescence of the modified enzyme due to an inner-filter effect associated with the absorption of the covalently bound DSB residues, and (ii) possible breakdown of the DSB-tryptophan residues under acidic conditions of hydrolysis with regeneration of tryptophan. The first possibility would explain a high figure for the extent of modification obtained from the fluorescence measurements while the second would explain a low figure derived from the amino acid analysis.

The effect of DSB modification on the conformation of MRS has also been investigated by means of circular dichroism (CD) studies. The unmodified enzyme has a CD pattern which is characteristic of globular proteins having a moderate  $\alpha$ -helix content. DSB-modified enzyme did not differ significantly from the native enzyme in its CD pattern. Thus, although there may be local conformational perturbations resulting from reaction of DSB with methionyl-tRNA synthetase, there seems to be no major disruption of the secondary and, probably, the tertiary structure of the enzyme.

### c. Substrate-binding studies

In order to discern whether the inhibition of MRS by DSB is due to interference with the ability of the enzyme to form complexes with its substrates or to direct reaction of DSB with a residue at the active site of the molecule, we have endeavored to measure directly the binding of substrates to the inhibited enzyme. To date we have examined only the interaction of the cognate transfer RNA with the enzyme. This complex is more difficult to observe than those of the much smaller substrates, methionine and ATP. One method for the measurement of complexes between tRNA and aminoacyl-tRNA synthetases has been the nitrocellulose filtration technique of Yarus and Berg (8). However, we have found the efficiency of this method to be unacceptably low and thus turned to other possibilities. As has been shown by Eldred and Schimmel (9), complexes of isoleucyl-tRNA synthetase and its cognate tRNA dissociate

in the course of elution through gel filtration columns. Thus one cannot isolate a complex which is at equilibrium with unassociated enzyme and tRNA. However, the Hummel-Dreyer technique (10), in which the ligand is uniformly distributed through the column, does allow one to isolate a complex which is at equilibrium with the unbound ligand. For the present work we used unfractionated tRNA as the source of tRNA<sup>Met</sup>. By doing so we are assured that non-specific binding of the cognate tRNA is minimized. The amount of tRNA complexed with the enzyme is determined by assaying the individual fractions for total tRNA<sup>Met</sup> using a standard aminoacylation assay. As shown in the Figure, when native MRS is passed through such a column, a peak of met acceptance capacity is observed in fraction 10, the position at which enzyme alone elutes. When DSB-inhibited enzyme (HNB-MRS, in the Figure) was passed through the column, a peak of comparable magnitude is observed. This indicates that inhibition of the capacity of MRS to aminoacylate tRNA<sup>Met</sup> is not due to a loss of the ability to bind the tRNA. Studies of the binding of methionine and ATP to DSB-inhibited MRS have not been completed. Should it turn out that all substrates may be bound by the inhibited enzyme, we would have compelling evidence that this reagent acts at the active site of the enzyme.

## 2. Evidence that Dithiothreitol Interferes with the Binding of Methionyladenylate to MRS

Methionyl-tRNA synthetase is an enzyme which has sensitive sulphydryl groups which are necessary for the activity of the enzyme (1,11). Thus it is customary to utilize sulphydryl compounds such as  $\beta$ -mercaptoethanol or dithiothreitol in experiments to prevent oxidation of the enzyme's SH groups. We have discovered that methionyladenylate will form a complex with MRS which can be isolated from excess methionyladenylate on a small Sephadex column using a buffer with no sulphydryl compound. Surprisingly, when the experiment was repeated with 10 mM dithiothreitol (DTT) in the buffer, no complex could be detected.

There are several plausible mechanisms by which this effect of DTT might occur: a direct interaction with either the methionyladenylate or the enzyme which would prevent complex formation, or a chemical reaction with the methionyladenylate resulting in either hydrolysis or the formation of a thiol ester between methionine and DTT. Experiments were carried out in which tRNA<sup>Met</sup> and fresh MRS were utilized to measure the amount of activated methionine present after methionyladenylate had been incubated with DTT, with or without MRS being present. These experiments indicated that failure to observe a complex between methionyladenylate and MRS using the Sephadex column was not due to hydrolysis of the adenylate. At present the most likely mechanism for the effect of DTT is a direct interaction with the methionyladenylate with the formation of either a non-covalent complex or with reaction to form the thiol ester. We are presently attempting to determine whether a thiol ester is formed by means of differential charcoal adsorption techniques or by thin-layer chromatography. A finding that a methionyl thiol ester may serve as a donor of methionine in the aminoacylation of tRNA<sup>Met</sup> would suggest that the aminoacyladenylylate is not an obligate intermediate in the aminoacylation reaction and that the active site of the enzyme can attack certain types of chemical bonds other than the ester bond of the aminoacyladenylylate.

We regret that Dr. Coleman is leaving to take another, more permanent position at this time when interesting, provocative results are beginning to turn up--such as the possibility of the thiol ester intermediate mentioned above.

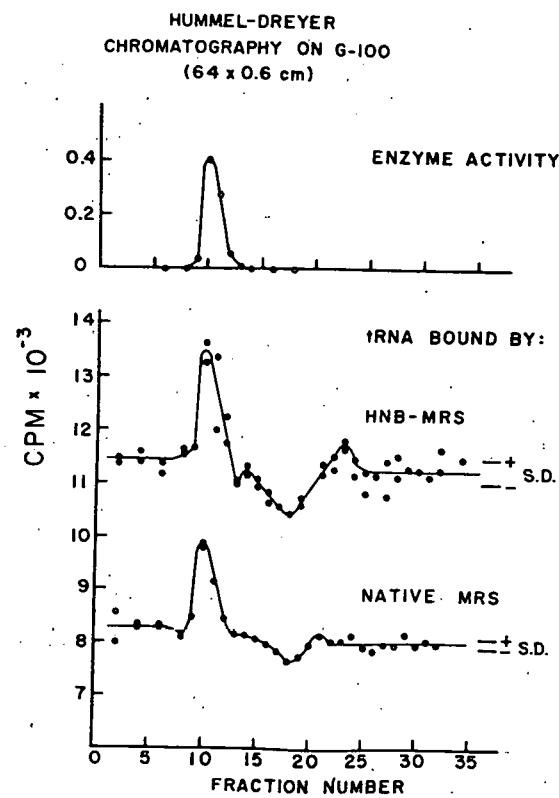


Figure 1. Quantitative determination of tRNA<sup>Met</sup> binding capacity of native and DSB-modified methionyl-tRNA synthetase.

## 3. References

1. Coleman, D.L. and Zamecnik, P.C. Fed. Proc. 32:460, 1973.
2. Coleman, D.L. Fed. Proc. 34:485, 1975.
3. Horton, H.R. and Koshland, D.E., Jr. in *Methods in Enzymology XXV*, C.H.W. Hirs and S.N. Timashoff, eds., Academic Press, New York, pp. 468-482, 1972.
4. Cassio, D. and Walter, J.P. Eur. J. Bioch. 20:283, 1971.
5. Spencer, R.L. and Wold, F. Anal. Bioch. 185, 1969.
6. Blangwet, S., Fayat, G., Waller, J.P., and Iwatsubo, M. Eur. J. Bioch. 24:461, 1972.
7. Bruton, C.J., Jakes, R., and Koch, G.L.E. FEBS Letters 45:26, 1974.
8. Yarus, M. and Berg, P. J. Mol. Biol. 28:479, 1967.
9. Eldred, E.W. and Schimmel, P.R. Anal. Bioch. 51:229, 1973.
10. Hummel, J.P. and Dryer, N.J. Biochem. Biophys. Acta 63:530, 1962.
11. Cassio, D. Eur. J. Bioch. 4:222, 1968.

D. Influence of Metabolites on Lysyl-tRNA Synthetase Formation in  
E. coli - I. N. Hirshfield

During the past 12 to 18 months we have concentrated most of our effort on purifying lysyl-tRNA synthetase from E. coli grown under specified conditions. These conditions are minimal medium in the absence or presence of 20 mM L-alanine or 3 mM glycyl-L-leucine. These conditions were chosen because numerous experiments in this laboratory have shown that L-alanine and small, hydrophobic, leucine peptides such as glycyl-L-leucine when present in minimal medium can stimulate the activity and apparently the amount of lysyl-tRNA synthetase in wild-type cells (2-3 fold) and in mutants up to 50-fold (1-4).

Therefore we have grown wild-type E. coli K-12 in minimal medium, minimal medium plus alanine or minimal medium plus glycyl-L-leucine. We also grew one mutant in minimal plus 20 mM L-alanine, but did not grow it in minimal medium alone because on the scale we used (30 liters) we felt the amount of enzyme available in this strain (1-3% of wild-type) would be too small to work with. The lysyl-tRNA synthetase was purified to 70-90% of homogeneity from each of these cultures. On the basis of physical and kinetic studies conducted with these preparations we have reached two major conclusions. First, the molecular weight of lysyl-tRNA synthetase from E. coli is at least 135,000-140,000 daltons in contrast to reports from the literature that found the molecular weight of this E. coli synthetase to be between 80,000-110,000 daltons (5-7). Not surprisingly our evidence indicates that lower molecular weight activity fragments can be formed as the result of proteolysis. In fact, quite recently we have been able to chromatographically resolve both a 135,000 and a 100,000 form of the synthetase by purification under the proper conditions.

The second major observation we made in these studies was that growth of the wild-type enzyme in the presence of L-alanine or glycyl-L-leucine resulted in a change in the properties of lysyl-tRNA synthetase. This was reflected in the fact that the synthetase purified from cells grown in the presence of the metabolites was more stable to thermal and urea denaturation and had different kinetic constants for the substrates lysine, ATP, and tRNA when compared to the synthetase from cells grown only in minimal medium. Comparison of the peptide grown synthetase to the minimal medium synthetase showed the former was also more resistant to trypsin inactivation and dilution inactivation, that is in dissociation into inactive subunits upon dilution, compared to the latter form of the enzyme. The apparent difference in the  $K_d$  of these two forms was 8-fold.

These results could plausibly be given two interpretations. Either the metabolites induce the formation of a second (or even a third?) lysyl-tRNA synthetase, or the metabolites by an unknown mechanism cause an alteration in the properties of the minimal medium form of the enzyme. At present we favor the latter interpretation although we believe we have to keep an open mind. One reason why we favor the alteration theory is based on experiments comparing lysyl-tRNA synthetase from the mutant strain grown plus alanine with the wild-type enzyme grown with alanine. Although these synthetase preparations share some properties in common there are qualitative and quantitative differences between them. This might not be expected if the same synthetase were being induced by alanine.

If as we suspect the altered properties of the synthetase are due to a modification of the enzyme, this would suggest a structure-function relationship between alteration of the enzyme's structure and its rate of synthesis. It is thus possible that this synthetase is autoregulatory. The fact that the peptide glycyl-L-leucine can influence the synthesis of lysyl-tRNA synthetase is quite intriguing, for peptides (as hormones) have well-established roles in modulating macromolecular synthesis in eukaryotes, but not in prokaryotes.

### References

1. Hirshfield, I.N. and Zamecnik, P.C. (1972) *Biochim. Biophys. Acta* 259, 330.
2. Hirshfield, I.N. and Buklad, N.E. (1973) *J. Bacteriol.* 113, 167.
3. Buklad, N.E., Sanborn, D., and Hirshfield, I.N. (1973) *J. Bacteriol.* 116, 1477.
4. Hirshfield, I.N., Yeh, F.-M., and Sawyer, L.E. (1975) *Proc. Nat. Acad. Sci. USA* 72, 1364.
5. Marshall, R.D. and Zamecnik, P.C. (1969) *Biochim. Biophys. Acta* 181, 454.
6. Stern, R. and Peterkofsky, A. (1969) *Biochemistry* 8, 4346.
7. Rymo, L., Lundvik, L., and Lagerkvist, U. (1972) *J. Biol. Chem.* 247, 3888.

E. Development of a Chemical Sequencing Technique that Will Facilitate Sequencing of mRNAs, Particularly Oncogenic Viral Messenger RNAs - D. Schwartz

1. Introduction
2. Investigations of Derivatives of the Type II and III (Figure 1) in Order to Develop Sequencing Methods Employing Post-labeling Techniques
3. General Strategy: Isolation of the oligonucleotide in a pure form after each cycle of degradation by chromatography on DEAE cellulose at acid pH
4. Use of Activated Charcoal to Separate Labeled Nucleoside Dialdehydes from Salts and Labeled Contaminants
5. Fractionation and Detection of the Labeled Dialcohol Nucleoside Derivatives by Thin Layer Chromatography and Fluorography
6. Use of DNA Oligonucleotide or Polynucleotide as Carrier for the RNA Oligonucleotide During Sequence Analysis
7. Adaptation of the Post-Labeling Method of Sequence Analysis to a Solid Support System

For the sake of clarity most of this report has been included in the initial part of Dr. Schwartz' section of the Proposal (Section 5.II.(E))

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1. Introduction

In 1953 Whitfeld and Markham (1) and Brown et al. (2) suggested that a method for the sequence analysis of polyribonucleotides might be perfected by studying the successive chemical removal of nucleotides from their 3'-terminals. The procedure would involve (i) the periodate oxidation of the terminal cis-glycol group of the polynucleotide chain, (ii) the removal of the terminal nucleoside by a base-catalyzed  $\beta$ -elimination reaction, and (iii) the removal of the terminal phosphate group so formed by phosphatase, leaving the polynucleotide chain in a condition suitable for a second cycle degradation. In 1960 Yu and Zamecnik (3) and Khym and Cohn (4) reported that the  $\beta$ -elimination reaction was catalyzed by primary amines, and it was demonstrated by Yu and Zamecnik that the stepwise method of degradation could be used to sequence the terminal region of tRNA. More recently Weith and Gilham (5,6) and Uziel et al. (7,8) have made significant improvements and have sequenced the 3'-terminal regions of certain RNA bacteriophages and tRNA, respectively. Since the time of its discovery, the stepwise method of degradation has been studied in many laboratories and, in all cases, up until 1972, primary amines have been used to catalyze the elimination reaction and, in most cases, the reaction has been carried out in the presence of excess periodate carried over from the oxidation step. Under these conditions the product arising from the oxidized terminal nucleoside is the purine or pyrimidine base.

In 1972 it was reported by Schwartz and Gilham (9) that if the excess periodate is removed after the oxidation of an oligonucleotide and if the elimination reaction is carried out in the absence of a primary amine, a product is formed which is an unsaturated nucleoside dialdehyde of the type II (Figure 1). This derivative is highly reactive and can be readily condensed with a primary amine to form a morpholinium derivative of the type IV, or reduced with borohydride to form a stable unsaturated nucleoside dialcohol of the type III.

The derivatives represented by type II and III were reported to offer two different approaches to the labeling of the nucleoside dialdehyde resulting from the  $\beta$ -elimination reaction, and it was envisioned that these new reactions could provide a method for the sequence analysis of extremely small amounts of unlabeled RNA oligonucleotide.

Since the time of the above study, Dr. Schwartz has been working in these laboratories and has been extending his previous work with Dr. Gilham to develop an improved method for the sequence analysis of subnanomolar amounts of unlabeled oligonucleotide. A method of sequence analysis has been developed which is several orders of magnitude more sensitive than the previously developed procedures of sequence analysis by stepwise degradation and which consequently will allow the sequence analysis of subnanomolar or picomolar amounts of an RNA oligonucleotide. In addition, it is anticipated that this new procedure can be adapted to a new and novel solid support system of sequence analysis which is capable of being automated. Such a system then would allow the rapid sequencing of extremely small amounts of RNA in much the same way that the Edman sequenator allows the rapid sequencing of polypeptides.

## 2. Investigations of Derivatives of the Type II and III (Figure 1) in Order to Develop Sequencing Methods Employing Post-labeling Techniques

In these laboratories we initially studied extensively the derivatives of type II and III (Figure 1) in order to determine which class of derivative was most suitable for use in a post-labeling method of sequence analysis. The use of tritium- or carbon<sup>14</sup>-labeled primary amines to produce labeled derivatives of type II proved to be unsatisfactory, since these derivatives are unstable at pH < 7 and therefore are not easily fractionated by standard chromatographic methods. In contrast, the derivatives of type III are very stable in acidic and basic media and thus can be readily analyzed by chromatographic methods previously developed for nucleoside fractionations. An additional advantage is that this type of derivative may be radioactively labeled to a high degree since tritiated borohydride of extremely high specific activity is commercially available.

## 3. General Strategy: Isolation of the Oligonucleotide in a Pure Form After Each Cycle of Degradation by chromatography on DEAE Cellulose at Acid pH

The strategy for the sequence analysis of subnanomolar amounts of oligonucleotide by stepwise chemical degradation is shown in Figure 2. It is to be noted that the most important step in the sequence of events constitutes the re-isolation of the oligonucleotide in a pure state after each cycle of degradation. We have found that this may be done rapidly by chromatography of the reaction mixture on a small column (0.5 x 2 cm) of DEAE cellulose at pH 5 as shown in Figure 2. At this pH and under conditions of low salt concentration, the oligonucleotide is selectively and quantitatively adsorbed while alkaline phosphatase (isoelectric point at pH 4.5), radioactive contaminants, and the radioactive nucleoside derivative are eluted through the column. Following a short elution with 0.05M triethylammonium bicarbonate (TEC) to convert the column to the bicarbonate form, the oligonucleotide is eluted with a small volume of 1M TEC and desalts by evaporation to dryness several times. The oligonucleotide is subjected then to a second cycle of degradation. The radioactive nucleoside derivative produced during the first cycle of degradation is

isolated from the low salt DEAE column effluent and identified by the procedures described below. (Note: It is essential that the alkaline phosphatase is not carried into subsequent cycles of degradation since the highly reactive nucleoside dialdehyde I (Figure 1) will react with the lysine residues in the enzyme to produce morpholinium derivatives of the type II).

#### 4. Use of Activated Charcoal to Separate Labeled Nucleoside Dialdehydes from Salts and Labeled Contaminants

At the termination of each cycle of degradation the radioactive nucleoside dialcohol, eluted from the DEAE column in the low salt wash, is separated from salts and radioactive contaminants by adsorption to activated charcoal followed by desorption in a salt-free form. We have modified an existing method (10) to develop a procedure which separates the dialcohol from >95% of the non-nucleoside contaminants labeled by tritiated borohydride and produces 85-90% yields of completely desalting nucleoside dialcohols in a form suitable for direct analysis by thin layer chromatography. (Note: Desalting of nucleoside dialcohols by published methods of carbon adsorption are not satisfactory since the procedures do not completely remove salt. Consequently the nucleoside dialcohols streak extensively during analysis by thin layer chromatography).

#### 5. Fractionation and Detection of the Labeled Dialcohol Nucleoside Derivatives by Thin Layer Chromatography and Fluorography

The derivatives of uridine, cytidine, adenosine, and guanosine, having structures corresponding to the type III (Figure 1), may be fractionated by the thin-layer chromatography methods developed by Randerath (11). The tritiated derivatives are detected by fluorography or by liquid scintillation counting and therefore labeled nucleoside dialcohol derivatives can be detected in the subnanomolar or picomolar range. (In practice, at the end of each cycle of degradation, the appropriate carrier nucleoside dialcohols are added to the reaction mixture. These are carried then through the DEAE chromatography step, the desalting step, and the subsequent analytical steps).

#### 6. Use of DNA Oligonucleotide or Polynucleotide as Carrier for the RNA Oligonucleotide During Sequence Analysis

The direct sequence analysis of subnanomolar or picomolar amounts of oligonucleotide by the stepwise method would be operationally difficult, if not impossible, due to irreversible adsorption of the extremely small amounts of the nucleotidic material to surfaces such as the walls of the reaction vessel or the DEAE cellulose. To circumvent this problem we have prepared a mixture of DNA oligonucleotide which is used as a carrier with the RNA during its sequence analysis. We have found experimentally that the DNA oligonucleotides do not interfere with the sequence analysis of extremely small amounts of RNA oligonucleotide but do function ideally as carrier since they behave physically in the same way as RNA oligonucleotides but chemically are inert to the cyclic degradations initiated by periodate oxidation. In practice the DNA mixture, which should consist ideally of oligonucleotides of random composition and length, is added to the RNA oligonucleotide at the beginning of the first cycle of degradation and is carried along with the RNA throughout all subsequent cycles.

A series of semi-random and random DNA oligomers have been generated by depurination and by pancreatic DNase digestion of calf thymus DNA, respectively. In the case of pancreatic DNase digestion, reproducible conditions have been found for the preparation of a series of stock solutions containing oligonucleotide mixtures of various specific average chain length. Thus, if the approximate length of the RNA oligonucleotide to be sequenced is known, it is possible to add an ideal mixture of DNA carrier (composed of deoxyoligonucleotides equal to or lower than the chain length of the oligoribonucleotide) to the RNA such that losses of the RNA during the subsequent sequence analysis are negligible.

### 7. Adaptation of the Post-Labeling Method of Sequence Analysis to a Solid Support System

We have attempted to adapt the post-labeling stepwise method of degradation described above to a solid support system previously described by Weith and Gilham (6) for the sequence analysis of optical density amounts of oligonucleotide. Unfortunately, for reasons which will not be discussed here, we have had little success on this endeavor. As a result we are now concentrating our efforts on the development of a new and novel solid support system.

Our strategy is to develop a reversible anion exchange resin which will function as an anion-exchange resin at pH 5 but will have no anion exchange capacity at pH 8.5. Such a resin would be ideal for our previously described studies since it could be substituted for the DEAE cellulose in the manual method of sequence analysis (Figure 2). For example, after completion of the first cycle of degradation, the reaction mixture could be acidified to pH 5, diluted, and passed through the reversible anion-exchanger (which is positively charged at pH 5). The oligonucleotide would be adsorbed and the radioactive nucleoside dialcohol, phosphatase, and radioactive contaminants would not be retained. Following a quick wash, the positive charges and thus the anion-exchange capacity could be removed from the resin by elution with the appropriate buffer at pH 8.5. The oligonucleotide could be washed directly into a reaction vessel where it would be carried through a second cycle of degradation. The process of degradation, adsorption, and desorption would be repeated until the oligonucleotide was completely sequenced.

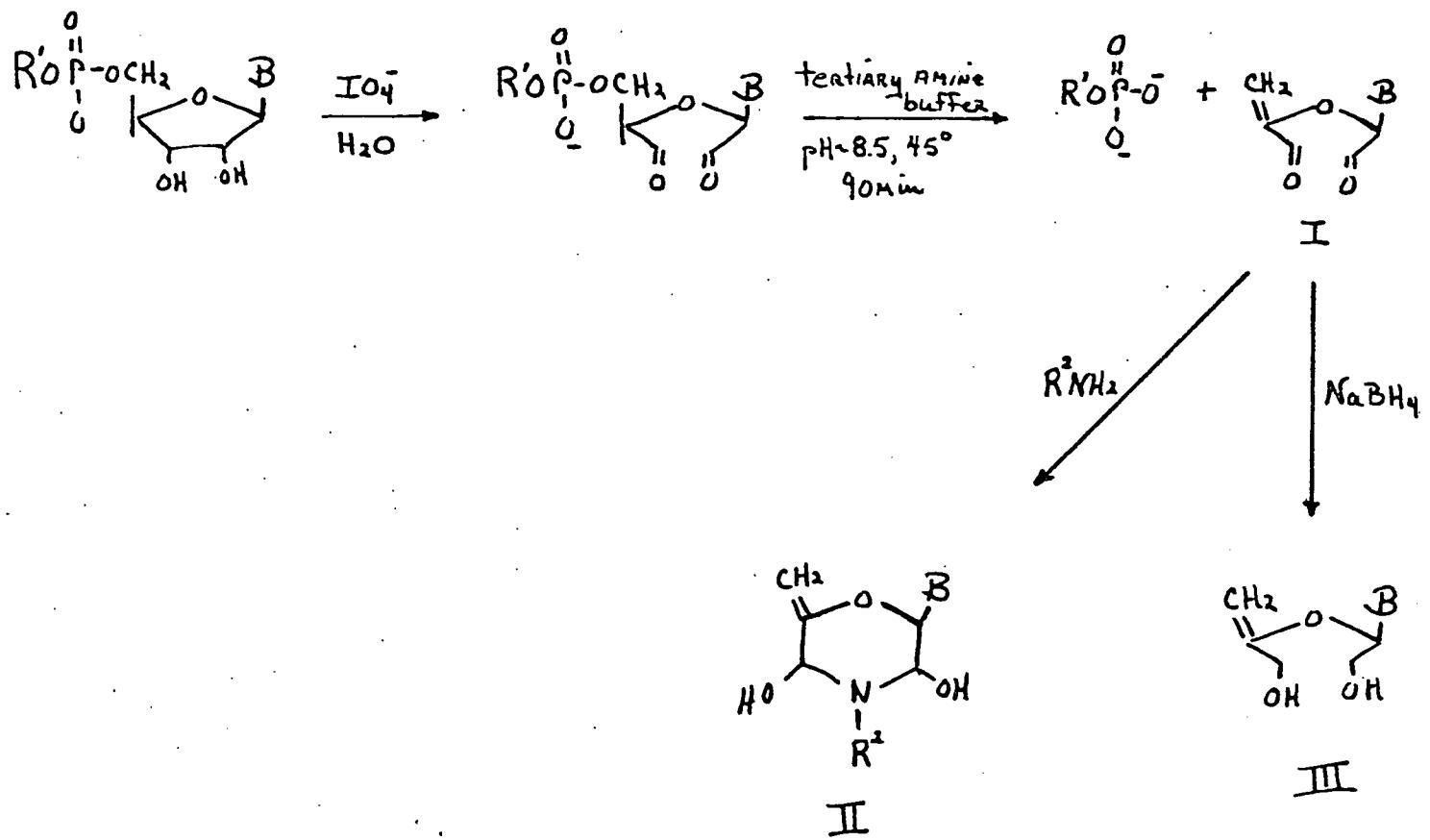
The advantages of such a solid support system are that (i) the method would eliminate the time consuming desalting step in the manual procedure, (ii) the method would be independent of the chain length of the oligoribonucleotide being sequenced, and (iii) the method could be automated.

After many trials we have been able to synthesize a resin which has the above properties. A polyacrylamide resin was converted to the hydrazide form by the method of Inman and Dintzis (12), treated with nitrous acid to form the azide, and then treated with histamine as shown in Figure 3. Under these conditions, a resin is formed which has the reversible anion-exchange properties shown in Figure 4 and more specifically exhibits a pka of 6 and 7.3 in water or 0.5M sodium chloride, respectively. Further studies have established that, under conditions of low salt concentration (0.05M), the resin quantitatively adsorbs oligonucleotides at pH 5 and quantitatively desorbs them at pH 8.5. The same success, however, has not been observed with tRNA which is adsorbed quantitatively at pH 5, but can only be 70% desorbed (low salt) at pH 8.5. This problem of tRNA desorption is believed to be due to the extremely high substitution of histamine on the polyacrylamide resin, and it is expected that lower histamine substitution of the resin will allow tRNA to be quantitatively adsorbed and desorbed under conditions of very low salt concentration.

We are in the process now of synthesizing polyacrylamide resins which are less substituted with histamine and are hopeful that the new resins can be used to develop a solid support system which will allow the rapid sequencing of subnanomolar and possibly picomolar amounts of an oligoribonucleotide.

### References

1. Whitfeld, P.R. and Markham, R. *Nature* 171:1151, 1953.
2. Brown, D.M., Fried, M., and Todd, A.R. *Chem. and Ind.* 352, 1953.
3. Yu, C.-T. and Zamecnik, P.C. *Biochim. Biophys. Acta* 45:148, 1960.
4. Khyrn, J.X. and Cohn, W.E. *J. Am. Chem. Soc.* 82:6380, 1960.
5. Weith, H.L. and Gilham, P.T. *Science* 166:1004, 1969.
6. Weith, H.L. and Gilham, P.T. 160th Nat. Meeting of Am. Chem. Soc., Chicago, Illinois, Sept., 1970, Abstract Biol. 18.
7. Uziel, H., Starken, J.W., Evelergh, J.W., and Johnson, W.F. *Clin. Chem.* 17:740, 1971.
8. Uziel, M. and Weinberger, A.J. *Nuc. Acids Res.* 2:469, 1975.
9. Schwartz, D.E. and Gilham, P.T. *J. Am. Chem. Soc.* 94:8921, 1972.
10. Mandeles, S. and Kamen, H.O. *Analyt. Biochem.* 17:540, 1966.
11. Randerath, K. and Randerath, E. *Proc. Nucl. Acid Res.* 2:796, 1971.
12. Inman, J.K. and Dintzi, H.M. *Biochemistry* 8:4075, 1969.

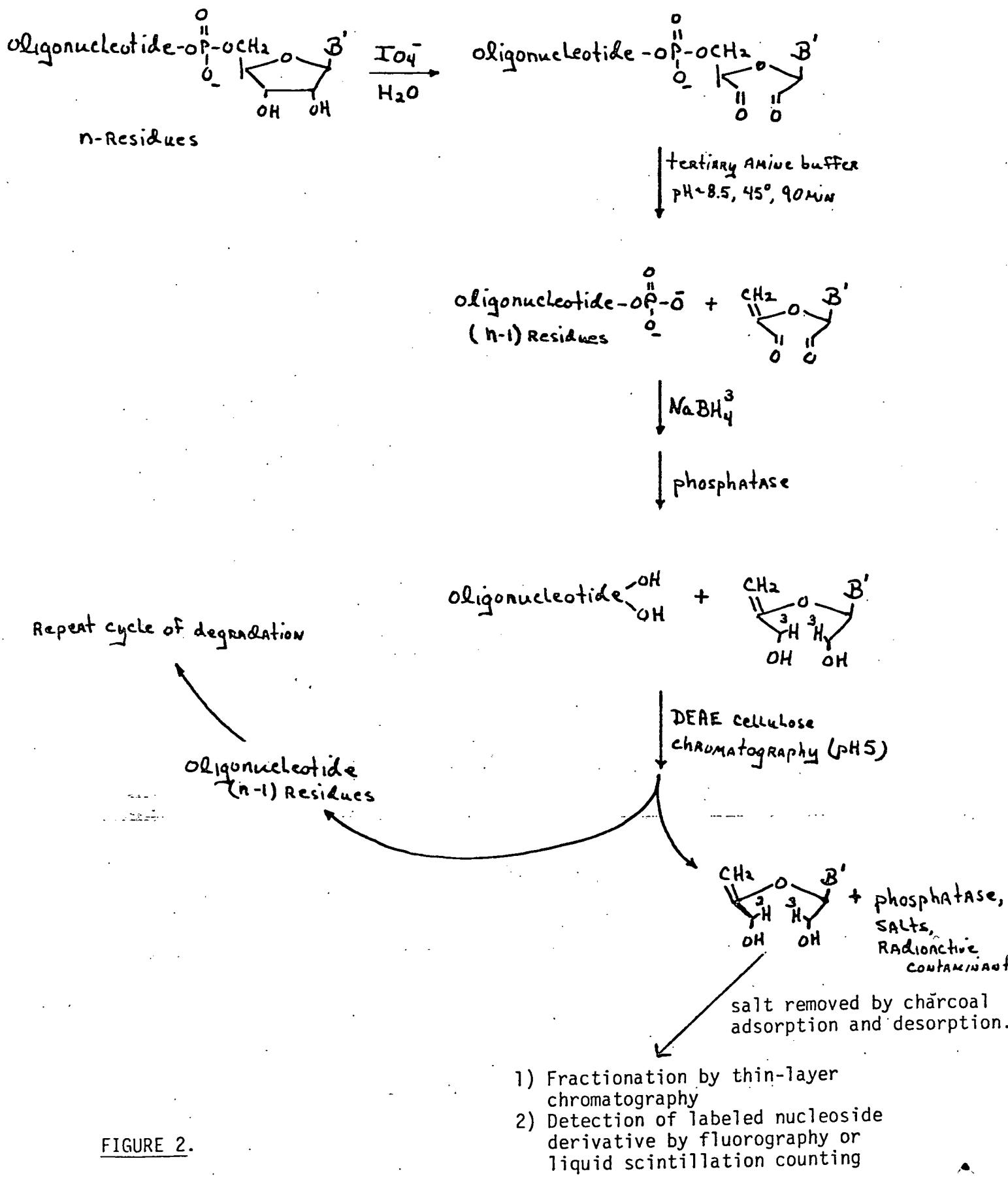


$\text{R}^1$  = H or nucleoside-3'

$\text{R}^2$  = H or alkyl group

B = purine or pyrimidine base

FIGURE 1



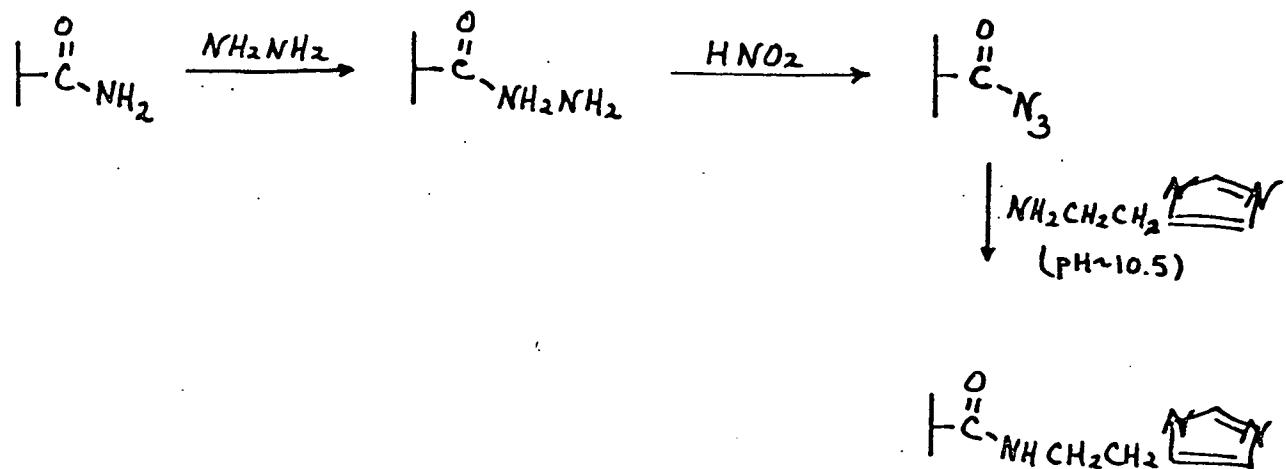


FIGURE 3. Synthesis of polyacrylamide-histamine resin.

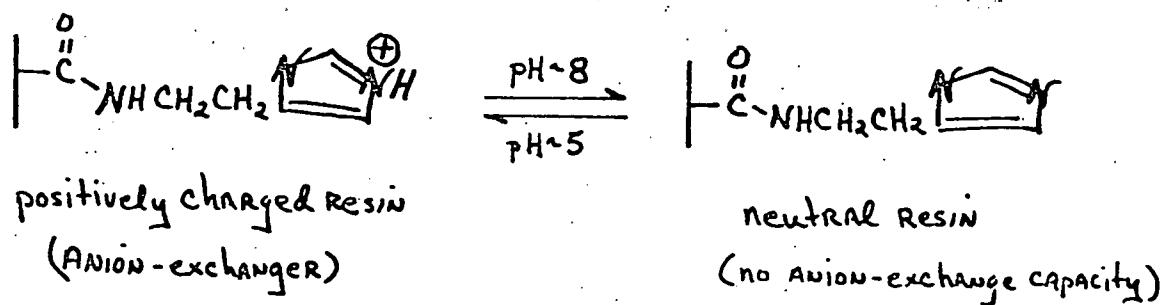


FIGURE 4. Reversibility of anion-exchange capacity of polyacrylamide-histamine resin.

### III. Publications

1. Hirshfield, I.N., Yeh, F.-M., Sawyer, L.E. Metabolites influence control of lysine transfer ribonucleic acid synthetase formation in Escherichia coli K-12. *Proc. Nat. Acad. Sci. USA* 72:1364-1367, 1975.
2. Hirshfield, I.N. and Price, M.B. Utilization of selected leucine peptide amides by Escherichia coli. *J. Bacteriol.* 122:966-975, 1975.
3. Rapaport, E., Svihovec, S., and Zamecnik, P.C. Relationship of the first step in protein synthesis to ppGpp: formation of A(5')ppp(5')Gpp. *Proc. Nat. Acad. Sci. USA* 72:2653-2657, 1975.
4. Heine, U.I., Weber, G.H., Cottler-Fox, M., Layard, M.W., Stephenson, M.L., and Zamecnik, P.C. Analysis of oncornavirus RNA subunits by electron microscopy. *Proc. Nat. Acad. Sci. USA* 72:3716-3720, 1975.
5. Rapaport, E., Berkley, P.D., and Bucher, N.L.R. Charcoal adsorption assay for measurement of colchicine binding and tubulin content of crude tissue extracts. *Analytical Biochem.* 69:92-99, 1975.
6. Zamecnik, P.C. Protein synthesis - early waves and recent ripples. *Ochoa Symposium*, J. Oro Ed., in press.
7. Rapaport, E. and Zamecnik, P.C. A new chemical procedure for post-isolation <sup>32</sup>P-labeling of ribonucleic acids at their 5'-ends. *Proc. Nat. Acad. Sci. USA* 72:314-317, 1975.

### ABSTRACTS

1. Coleman, D.L. Inhibition of methionyl-tRNA synthetase of E. coli by dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide (HNB). *Federation Proc.* 34:485, 1975.
2. Hirshfield, I.N., Yeh, F.-M., and Zamecnik, P.C. Apparent modification of lysyl-tRNA synthetase *in vivo* by L-alanine in Escherichia coli. *Federation Proc.* 34:486, 1975.
3. Weber, G.H., Heine, U.I., Cottler-Fox, M., Stephenson, M.L., and Zamecnik, P.C. Visualization of oncornavirus subunits by electron microscopy. *Sci. Proc. Am. Assoc. Cancer Res.*, San Diego 16:25, 1975.