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# PROTEIN SYNTHESIS AND ITS REGULATION:

# A BACKGROUND STUDY RELATED TO THE BIOLOGICAL EFFECTS OF RADIATION

Progress Report

for Period July 1, 1976 - August 31, 1977

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ANNUAL REPORT

UNITED STATES ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION

CONTRACT NO. EY-76-S-02-2404 (Formerly AT(30-1)-2643)

July 1, 1976 - August 31, 1977

# PROTEIN SYNTHESIS AND ITS REGULATION:

# A BACKGROUND STUDY RELATED TO THE BIOLOGICAL EFFECTS OF RADIATION


## Abstract

This contract has for many years been the principal support of our work on delineation of the steps involved in protein synthesis, and the role of transfer and messenger RNAs in the translation process. During the past year we have studied the mechanisms by which an oncogenic RNA virus modifies the growth process, and have begun to elucidate the role a novel dinucleotide, discovered in these laboratories, plays in rapidly growing cells in tissue culture.



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## 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) $Ap_4A$ 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. We also developed (i.e., the Randeraths did in our laboratories, supported under these auspices) polyethyleneimine cellulose thin-layer chromatographic separations of nucleotides in the mono- and oligonucleotide range.

Our most exciting finding of the current year (cf. the description in Science News 111:247, April 16, 1977) (Figure 1) was the sequencing of 21 nucleotides inside the poly(A)terminus of Rous sarcoma virus (RSV) 35S RNA. To our surpirse, on comparing this sequence with that being contemporaneously and independently worked out at the 5' end of the same RSV 35S RNA by Haseltine, Maxam, and Gilbert across the river from us at Harvard, *we found that there was a stretch of 21 identical nucleotides at both ends of this same molecule,* separated by a distance of 10,000 ribonucleotides.

Since it has been predicted and also observed by electron microscopy that a circular DNA intermediate occurs prior to integration of the retroscribed DNA of RNA oncogenic viruses, this unexpected finding of terminal nucleotide redundancy provides a basis for the postulation of this intermediate. It also leads to the new and exciting possibility of designing a competitive nucleotide virus inhibitor, as discussed in our current proposal.

We have used the 35S RNA of another oncogenic virus--the avian myeloblastosis virus (AMV)--for studies of the in vitro translational potential of this type of RNA. The AMV RNA has been used for this investigation rather than the purer RSV RNA because the AMV is available in the much larger quantities requisite for detailed studies of the translation step in protein synthesis, as directed by oncogenic viral RNA. Cell-free translation of AMV viral specific proteins has now been demonstrated, and factors which determine selectivity of the message translated are being investigated.

Diadenosine 5',5'''-$P^1$,$P^4$-tetraphosphate was discovered in these laboratories a decade ago. It was first found to be formed in the back reaction of the initial step in protein synthesis, in an in vitro enzymatic system. We then looked for $Ap_4A$ in whole cell systems, and found it to be present in rat liver slices, ascites tumor cells, and in E. coli. Due to technical difficulties, however, it was hard to pursue its metabolic properties. During the past year we have taken up the search for a possible regulatory role for $Ap_4A$ in mammalian cells in tissue culture, with the help of better column fractionation procedures and the use of $^3H$- and $^{32}P$-labeled $Ap_4A$ and precursors of high specific radioactivity. In brief, $Ap_4A$ levels are high ($\sim 3 \times 10^{-6}$M) where cell lines have a very rapid doubling time (9-11 hours), and the levels are low ($\sim 1 \times 10^{-8}$M) where cell doubling times are slow (24 hours or more). Thus the highest levels are found in rapidly growing malignant cells in tissue culture.

## II. Specific Reports

### A. Terminal Redundancy of the 3' and 5' Ends of Rous Sarcoma Virus 35S RNA - P. C. Zamecnik

The enclosed reprint from the March, 1977, PNAS (1) describes in detail the techniques used for determination of the sequence of ribonucleotides just internally adjacent to the poly(A) terminus of the 35S RNA of the Rous sarcoma virus. Under immediate predecessors of the present contract we established for the first time that a poly(A) segment existed as the 3'-termination piece of an oncogenic RNA virus, and did not occur again in an internal position. Armed with this knowledge, we then essayed to use an oligo $d(pT)_{10}$ piece as a primer, and employed a purified reverse transcriptase to copy the sequence of that segment of 35S RNA just internal to the poly(A) at the 3' end of the 35S RNA molecule. The results were initially ambiguous, when we employed AMV 35S RNA. We were conscious that AMV is a mixture of several closely related viruses, all of them ending in poly(A). We also had the feeling that there would be a piece of RNA just internal to the poly(A) which would be common to the AMV closely related family of viruses. In order to avoid the possibility of an early divergence of sequence, however, we shifted to use of Rous sarcoma virus - C strain, which came from a single, cloned viral ancestor, and which was kindly supplied by Dr. Eugene Bernstein of the University Laboratories, New Jersey.

By careful use of oligo $d(pT)_{10}$ primer, freed by us from the $dT_{8-12}$ oligomers which usually accompany synthesis of oligo $dT_{10}$, and by employment of a specially synthesized primer $dT(pdT)_8prG$, Drs. Schwartz and Weith were able to deduce the sequence 5'....(G)CCAUUUUACCAUUCACCACA-poly(A)3' as a principal sequence at the 3' end of 35S RSV RNA (1).

At the same time and completely independently, Haseltine, Maxam and Gilbert in the Biochemistry Department of the Faculty of Arts and Sciences at Harvard were engaged in sequencing the 5'-end of the 35S RNA of RSV (4). Maxam and Gilbert had recently devised a new, elegant chemical procedure for sequencing RNA (5), and unbeknown to us, had begun to use it on the other end of the same molecule as that which engaged our attention. We first learned of their work when we were talking to Dr. Gilbert about details of their new sequencing technique. It came as a complete surprise to both groups of investigators that the 21 nucleotides just internal to the Gppp- cap at the 5'-end of 35S RSV RNA were identical to our 21 nucleotides just internal to poly(A) at the 3'-end of the same molecule--separated by 10,000± nucleotides in between. As described in our proposal for the coming year, and in the paper of Haseltine, Maxam, and Gilbert (4), which was published back-to-back with ours in the March, 1977, PNAS, these findings can explain the circularization of the DNA intermediate following reverse transcription from input viral RNA, and prior to integration of the viral DNA information into the host genome.

The accompanying description from Science News (6) states in an objective way the implication of these findings (cf. Figure 1).

SCIENCE NEWS, Volume 111/April 16, 1977/No. 16, Page 247

# RNA tumor viruses: More insights

Because tumor viruses cause various kinds of cancers in animals and may possibly do so in humans as well, there is great need to learn more about how they can trigger disease in cells. For instance, during infection of a cell, an RNA tumor virus (a virus with a core of RNA as its genetic material) makes a DNA copy of its RNA. This DNA then becomes a closed circle and integrates into the cell's DNA before the life cycle of the virus can continue. A crucial question is: How can a linear RNA result in a circular DNA molecule?

A partial answer is provided in the March PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES by two separate research groups at Harvard Medical School and Harvard University. The investigators are William A. Haseltine, Allan M. Maxam and Walter Gilbert, and Dennis E. Schwartz, Paul C. Zamecnik and H. Lee Weith. They have found that the bases (chemical building blocks of nucleic acids) at both the left and right ends of viral RNA are virtually identical. Such identical sequences may help explain how a DNA copy of RNA can become circular.

When an RNA tumor virus enters a host cell, it carries two copies of linear viral RNA and several copies of the reverse transcriptase enzyme that it needs to make DNA copies of its RNAs. Also, attached to the left end of each RNA to help in transcription is a host-cell transfer RNA. As an enzyme starts to transcribe a viral RNA strand into a DNA copy, the tRNA primer is elongated into DNA. Then the enzyme goes on to make a DNA copy of some 100 bases at the extreme left end of the RNA. Using a new method that Maxam and Gilbert designed (SN: 4/2/77, p. 216), Haseltine, Maxam and Gilbert determined the sequence of this 100-base stretch. Meanwhile, Schwartz and his colleagues determined the sequence of bases at the right end. It turns out that the 21 bases at the extreme left end are also at its extreme right end. The only difference is that the stretch at the right end is followed by several A (adenine) bases.

So how do these identical ends help a linear DNA copy of a viral RNA to become circular? When DNA copying begins at the left end of the viral RNA, only one percent of the RNA is made into DNA. So how is a DNA copy of the other 99 percent made, since it must be made from the right, not from the left, end? Because the DNA transcription starts at the left end, the growing DNA chain must jump to the right end and then continue copying the rest of the RNA. And this is where the identical bases at the left and right ends probably come into use.

For instance, the growing DNA chain could float away from its RNA at the left end and pair up with another viral RNA at the right end for further elongation. Or a reverse transcriptase enzyme might digest some of the RNA from the RNA-DNA hybrid containing the 100 bases at the left end, revealing that DNA so that it can pair with the repeated sequence at the right end. Thus, as the DNA is brought around from the left end of the viral RNA to the right end, it also creates DNA circles. □

Figure 1

## B. The Translational Characteristics of Avian Myeloblastosis Virus 35S RNA - M. L. Stephenson

### 1. Introduction

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 a study of what happens when oncogenic RNA viruses insert themselves in 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 and often to produce tumors. Dr. Stephenson has been investigating cell-free protein synthesizing systems, particularly the requirements for the production of viral specific proteins made in response to specific RNA factors from the avian myeloblastosis virus in heterologous systems. One of the long-range plans was to attempt to identify the new production of the viral RNA directed DNA polymerase, the reverse transcriptase, by its enzymatic activity. 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 achieve this long-range goal it was first necessary to establish a reproducible cell-free incorporating system which responded to the addition of viral mRNA to produce viral specific proteins. For this reason the bulk of this section is concerned with our investigation of various cell-free systems and our attempts to elicit a reproducible and larger response to added viral RNA than has heretofore been obtained by us or others. It also covers our attempts to detect the formation of the reverse transcriptase in response to viral RNA in a heterologous system both by immunological methods and more specifically by looking for the production of its enzymatic activity using synthetic primers and templates. Although it is known that RNA from oncogenic RNA virus infected cells can produce viral specific proteins in certain cell-free systems, little has been done to pinpoint the properties of the RNA from *isolated virions* in heterologous cell-free systems.

The following describes work done by others and by us in establishing cell-free systems which are producing viral specific proteins in response to viral RNA.

The avian oncornaviruses contain several structural proteins. The major components are five internal polypeptides (p27, p19, p15, p12, and p10) and two glycoproteins found in the virus envelope (gp85 and gp37) in addition to the reverse transcriptase (RNA directed DNA polymerase) and in transforming viruses a region near the 3' end termed sarc (see review by Shapiro and August on cleavage events (7)). There are many precursor polypeptides which have been detected mainly in vivo using pulse chase experiments and which are not found in the intact virion in most cases. Figure 2 indicates the cleavage scheme of avian oncornavirus precursor protein pr 76 (Shapiro and August, adapted from Vogt (7,8)). The major viral proteins come from pr 76; however, even the envelope glycoproteins have large precursors in addition to non-glycosylated forms near p80. The reverse transcriptase has two active forms: an a unit of about 60,000 daltons and a b unit of about 100,000 daltons. a is believed to be a proteolytic cleavage product of b (9-11).

The same in vivo experiments (8) along with data from RNAase T resistant oligonucleotide studies (12-14) have enabled one to indicate the tentative gene order of avian sarcoma virus (Rous) (see Figure 2 (7)). The 5' end contains a methylated oligonucleotide. This has been demonstrated in our laboratories for AMV (3) and by Shatkin et al. for RSV (15). The in vivo pulse chase experiments have proved most helpful in determining the existence of polyproteins and in ascertaining the order of genes in the 35S viral RNA.

In contrast with the in vivo experiments the in vitro results indicate to date mainly the formation of viral specific proteins related to pr 76 which start near the 5' end of the 35S RNA. The other proteins have not been detected or if so in minute quantities (7).

If the total genome of 35S RNA were translated as a single precursor, a 350,000 dalton protein could be the primary translation product. There has been some evidence both for and against such a large precursor which if present has been detected in only very small amounts (7). The question is still in doubt as to the following types of mechanism of synthesis:

A. One primary translation product from the entire 35S RNA genome--i.e., one initiation site. This would be followed by rapid cleavage. There also could be selective degradation and variable sites of termination leading to different amounts of virus proteins under this hypothesis.

B. A second alternative could be that there are different sizes of oncornavirus mRNA molecules, each one making a specific viral protein. This is possible since there is evidence for 14 and 28S RNA molecules (16,17) in the 70S RNA from the virion as well as viral specific 20S RNA found in infected cell polysomes (18).

C. A third possibility is the presence of multiple initiation sites on a large RNA.

To date there is conflicting evidence both in vivo and in vitro as to exactly how the proteins except pr 76 are originated.

2. Cell-free protein synthesizing systems

The preceding introduction has been included to stress the complexity involved in attempting to identify viral specific proteins on SDS polyacrylamide slab gels. Unless the synthesis and cleavage are complete almost any size protein might be present in the heterologous cell-free systems. In spite of this fact much can be learned from such systems particularly through the use of antibodies directed against specific viral proteins.

Several laboratories have provided evidence that the RNA from oncogenic viruses has messenger activity in cell-free protein synthesizing systems (19-21) and have found that at least some of the polypeptides formed are antigenically related to the virus (22-31). A variety of systems have been used and some produce large polyproteins. Many of these have been from RNA extracted from polysomes of infected cells added to an heterologous system.

A recent paper by Salden and Bloemendal using a reticulocyte lysate indicated that AMV-RNA was translated into a precursor polypeptide of 76,000-80,000 daltons. Several smaller precursor polypeptides and the four major internal structural viral proteins were detected after immunoprecipitation with antiserum against the gs antigen of AMV (29). Such a cell-free system is apparently able to produce and cleave the precursor polypeptide, pr 76,000.

Almost all the cell-free systems, including ours, used the 70S RNA or heated 70S RNA of oncornavirus in cell-free systems composed of "infected" tissue culture cells, rabbit reticulocytes, ascites cells, or wheat embryo extracts. Following incubation with a labeled amino acid (usually $^{35}$S-methionine) the proteins were exposed to antisera made from the virus or specific oncornavirus proteins and after the addition of small amounts of carrier virus proteins the immune precipitates were collected. These viral specific proteins were washed and fractionated using SDS-polyacrylamide slab gel electrophoresis to separate the proteins. Radioautographs were made of the gels to locate the labeled protein bands. Considerable enhancement of detection of the bands was achieved by impregnating the gels with PPO in $Me_2SO$ and prefogging the film (32-33).

(For the sake of brevity and clarity all the representative Figures and Tables *of our experiments* have been placed at the end of this section, together with a short description for each).

For the most part we have used a wheat embryo system similar to that of Roberts and Paterson (34), using an S-30 fraction (sometimes pre-incubated) isolated from Sephadex G-25, or a dialyzed S-30 made according to Davies and Kaesberg (35). Our optimal conditions of KCl (40 mM), $Mg(Ac)_2$ (1.5 mM) corrected for amount taken up by ATP and GTP, Tris buffer at pH 7.5, spermine (80 mM), amino acids (.030 mM each), wheat embryo extract, time, temperature, and heated 70S RNA from AMV (4-8 μg/50 μl) have been elaborated for labeling of alkali-stable, TCA precipitable material using $^{35}$S-methionine or $^{3}$H-leucine. We have achieved 3-6 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 that achieved by some other mRNAs such as globin mRNA or TMV RNA (see Figure 3).

It is not sufficient to depend upon counts in protein as an accurate reflection of viral protein synthesis. We have routinely incubated 50 μl samples, precipitated, alkali treated, washed, and counted 10 μl and then applied the remaining 35 μl on SDS polyacrylamide slab gels (15 x 25 cm x 1.5 mm thick) in order to see the size of the labeled proteins. We have found this to be essential for several reasons: 1) to compare unknown bands with known viral proteins labeled with $C^{14}$ (36) or stained for proteins; 2) the added viral RNA has stimulated synthesis of some endogenous proteins in some cases, especially in the purified reticulocyte system; 3) the viral RNA markedly inhibits globin synthesis in the reticulocyte lysate and may inhibit other endogenous proteins by competition with the endogenous RNA (see Figure 4). Thach et al. (37) have recently indicated inhibition of globin synthesis encephalomyocarditis virus mRNA by competing with the endogenous globin mRNA for an initiation factor.

The 7-18 percent polyacrylamide SDS gradient gels are run overnight using the tris buffer system of Laemmli (38). The gels are treated with PPO in DMSO prior to drying and the film sometimes prefogged (32,33) in order to enhance the detection of the $^{35}$S-methionine in the proteins in radioautographs developed at -70°. Figure 5a indicates a stained gel using coomassie blue. Most of the stained proteins of the system do not show up in the radioautographs (Figure 5b) since we have chosen systems with a low endogenous incorporation.

Although the presence of viral RNA indicates the formation of different labeled proteins than are present in the control endogenous incorporation we rarely see bands that correspond exactly with AMV proteins (from lysed purified virus) run in adjacent or the same channel.

Immune precipitates have been made in many experiments using for the most part anti-AMV prepared from tween-ether disrupted virions or purified P30 proteins. The virus, AMV (BAI Strain A), the antisera to Tween-ether disrupted virions and to the purified P27 of AMV are supplied via the Virus Cancer Program from the Office of Program Resources and Logistics through the courtesy of Dr. Jack Gruber. The virus has been generously supplied as frozen pellets through the kindness of Dr. and Mrs. Beard of Life Sciences Research Laboratories, St. Petersberg, Florida. The antisera were sent to us by Dr. Roger Wilsnack of the Huntingdon Research Center, Brooklandville, Maryland. We have used pig antisera to Tween-ether disrupted AMV and goat antisera to the purified P27 protein from AMV.

In order to quantitate the antibody it was titrated against lysed AMV which had been labeled using $C^{14}$-formaldehyde and unlabeled borohydride (36). As indicated in Figure 6A and B almost all of the added AMV-$C^{14}$ was found in the antigen-antibody pellet. Although we are satisfied that the antigen is reacting with the antibody, there was a possibility that the antigen (AMV proteins) were being hydrolysed by proteolytic enzymes in the wheat embryo extract. Gels run on the antigen-antibody pellet made alone and in the presence of wheat embryo enzymes gave labeled bands that corresponded exactly with those of the labeled AMV proteins run separately. Therefore we feel that although we are getting many viral specific proteins made in response to added viral RNA the fact that they do not match the AMV proteins from the mature virion is not an artifact of the immunological procedure. Our results using both the wheat embryo and reticulocyte systems give evidence for the presence of many viral specific proteins, some of high molecular weight (unpublished experiments). These have not yet been critically identified as to whether they are precursor proteins or whether some are the proteins found in the intact virus. Both the reticulocyte lysate and the wheat embryo extract or the viral RNA may be lacking some factor(s) which prevent the system from making mature viral proteins. The proteins made in response to AMV RNA are in all size ranges (see Figure 5b). Experiments carried out with Dr. J. Gilbert using purified rabbit reticulocyte ribosomes plus factors indicate a 20-30 fold stimulation upon addition of heated AMV 70S RNA. A very recent experiment of Tse and Taylor (39) indicates that the wheat embryo system using purified rat liver albumin mRNA gave many anti-albumin immune specific proteins (such as we see) but that when the potassium or magnesium concentration is increased, above the optimum for total CPM incorporated, the immune specific counts are found in the specific protein albumin. Apparently at the lower concentrations of potassium or magnesium the viral specific proteins are *terminated prematurely*. We are currently investigating such conditions with AMV-RNA.

The pmoles of amino acid we find incorporated into proteins in response to added viral RNA in the cell-free systems is reproducible and we are satisfied that although the incorporation is fairly low the conditions are reliable enough for us to use these systems with AMV RNA. It is anticipated that the addition or possibly the removal of small linker and/or regulatory molecules found in the 70S RNA complex will stimulate the incorporation to the much higher levels achieved by some non-oncornavirus mRNAs (i.e. globin mRNA and TMV RNA).

We have not ruled out the classical interpretation that all the true mRNA is made once the virion RNA has entered the cell, been transcribed to DNA, integrated into the host DNA, and been transcribed by the cell RNA polymerase. There is no question, however, that cell-free protein synthesizing systems are able to make viral specific proteins using oncornavirus RNA as template, although there are few instances where these viral specific proteins are identical to proteins found in the mature virion (7). One of the directions of our research is to determine whether or not we can increase the mRNA activity of the virion and just what fraction of the 70S RNA complex is involved.

It is well known that optimal conditions for one type of mRNA may not be 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. It is obvious that there is a real need to determine the factors responsible for such enormous variations in response to the added mRNAs and our very recent findings with the reticulocyte cell-free system 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. In particular we would like to know whether the viral RNA as isolated is available to exert a role as mRNA or whether it is unable to serve as mRNA until blocking agents are removed.

Originally we chose the wheat embryo system because it is capable of making faithful proteins for certain mRNAs (40-45). Very recent evidence has indicated that the system can make the precursor proteins of growth hormone (46) but apparently does not contain the particular enzymes necessary for cleavage of the precursor protein(s). We are aware of the fact that specific "cleavage" enzymes may have to be added from other sources: wheat germ may in fact be a good system in which to study such factors. Its low ribonuclease activity, low endogenous incorporation plus the fact that the extract is said to contain all the RNA "capping" enzymes (47) make it a favorable choice at present. As mentioned above, however, the reticulocyte system under our conditions may have some advantages.

3. Studies with avian myeloblastosis virus RNA (AMV)

Frozen viral pellets are prepared specially for us by Dr. Beard, shipped in dry ice, and then thawed directly in a 1:1 mixture of buffer and phenol plus other RNase inhibitory ingredients thus minimizing the risk of nuclease activity on the RNA which we obtained by ethanol precipitation of the phenol extracts. We have published the first work showing that the 35S RNA of AMV terminates in adenosine at the 3'-end, that a poly(A) terminus is present, and that all the poly(A) is at that end of the molecule (2-3). We prepared some 35S RNA from heated 70S AMV RNA for Drs. Ursula Heine and George Weber to examine in the electron microscope. 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 (48).

We are also cognizant of the fact that the 35S RNA fraction prepared from the AMV is not a single uniform peak but contains subunits of lower varied molecular weight when isolated from DMSO-sucrose gradients. The extent that this RNA represents actual "processing" or random scission is discussed below. Because the high molecular weight RNA is very susceptible to ribonuclease activity, we have made vigorous efforts to prepare RNA free of such degradation.

Other laboratories have shown that high molecular weight RNA detected from 2-6 hour incubations with $P-^{32}$ phosphate in tissue culture-grown RNA viruses give a single uniform peak. This material, however, represents of the order of 1/10,000th or less of the amount of high molecular weight RNA obtained from infected chicks and cannot be obtained in the μg amounts required for cell-free translation. It has recently become well documented that RNA transcripts in cells are progressively shortened and modified with time, in their processing for eventual use as messenger RNA, tRNA, ribosomal RNAs, and other RNAs of as yet unknown function. It is our opinion that the total RNA of the AMV virion undergoes these varied modifications with time, in what has been known as the "maturing process", recognized by electron microscopy as a change in the electron dense region of the nucleoid area of the virion. Thus, in virions obtained from whole animal sources, there is at present an open question as to whether the increasing scissions in high molecular weight virion RNA may not be part of this maturation process. This is what our data with Dr. Heine and colleagues suggests--not random breaks in the 35S RNA, but specialized cuts by selective nucleolytic enzymes, for particular purposes as yet unknown to us. Thus in our view we are not suffering from random ribonuclease activity, but are recording in detail the size distribution of RNAs as they exist in the intact, mature AMV virion.

It is thought that processing occurs just as the virus is budding from the cells since viral proteins are not found within the infected cells. There are separate polyproteins coding for the gag region and the env areas on the genome. The RNA we obtain from intact virions from chick plasma has been processed and perhaps cannot be compared with that viral RNA found on polysomes of viral infected cells. One of the purposes of our studies is to answer this question.

Dr. H.P.J. Bloemers (49) has evidence in the oocyte using translation of fractions of mRNA from Rauscher virus infected cell polyribosomes off a 50 percent dimethylsulfoxide gradient that the size of mRNA coding for one of the internal proteins is actually about 14S. This evidence for complete translation of small viral protein from a small segment of the polycistronic 35S RNA indicates that at least *in the oocyte* the polycistron does not have to be intact for translation of a viral structural protein.

An interesting paper just published by G. Shanmugam (50) reports an endonucleolytic cleavage of MLV 35S RNA into two fragments by a microsome associated nuclease with evidence suggesting double stranded regions in the 35S RNA as the site of cleavage.

There is little difference whether AMV heated 70S RNA or 35S RNA isolated from sucrose gradients is used in the heterologous systems. Unheated 70S RNA is less efficient but not markedly so. Although the stimulation to date is not remarkable, it is reproducible and we are using the wheat embryo system and the rabbit reticulocyte systems to investigate various RNA fractions.

An experiment we carried out to determine possible sizes of mRNA from the 70S complex has indicated that small RNAs produce viral specific proteins. When pooled 70S RNA from a sucrose gradient fractionation was treated with 85 percent dimethylsulfoxide and the RNA fractionated on a DMSO-sucrose gradient so that reaggregation could not take place, the $A_{260}$ profiles seen in Figure 7 were obtained. RNA fractions were pooled as indicated and equal μg of RNA were assayed in the wheat germ system as shown in Table 1A. The results indicate that the RNA pool in the 10S area, and the 18-28S area were both more active than the 35S peak

pool on a μg basis. On a molar basis, however, they would have roughly similar activity. An earlier experiment using a different RNA preparation gave similar results. The proteins in an immune precipitate against anti-AMV had activity in all fractions except the 4S area. Gels of the labeled proteins indicated that the smaller RNA pools made smaller proteins, while the 35S RNA made all sizes. Further fractionation of the RNA pools into poly(A)+ and poly(A)- using oligo(dt) cellulose were carried out in order to see whether some of the smaller RNA might have come from the 3' end of the 35S RNA genome. Table IB indicates that both the poly(A)+ and poly(A)- fractions have mRNA activity. We have shown that all the poly(A) of the 35S RNA and 70S RNA is at the 3' end of the genome (3). Since translation starts at the 5' end of the molecule and some of the active RNA is in the 10S region containing poly(A) the active mRNA here is a small molecule, possibly originating from the 3' end of the 35S genome. This preliminary experiment indicates that some of the mRNA could have come from near the 3' end of the molecule and/or that there are small RNAs with mRNA activity in the mature 70S RNA complex. Much work needs to be done to characterize these smaller mRNA molecules. Salden et al. (28) have recently indicated the presence of small mRNA molecules in the Rauscher leukemia virus in heterologous cell-free systems.

We have many times noted that total viral RNA is inhibitory and have wondered whether small molecules present in it (including those in the 70S complex) might not interfere with the cell-free synthesis in response to 35S RNA. Various mixtures of the fractions isolated from the DMSO gradient showed that the 4S RNA area does inhibit to some extent whereas the other RNA fractions apparently do not. This experiments was carried out using a low input of RNA, 2 μg each, so that the combined RNAs would not exceed the plateau labeling of any single RNA. See Figure 8 for a labeling concentration curve of these individual RNA fractions and note that as with most mRNAs, high concentrations limit the reaction.

Experiments are in progress to isolate various fractions of AMV and to try to pinpoint the active fraction which is stimulating the incorporation into specific viral proteins. Further knowledge of the initiation site or sites on the genome RNA and the role of 5'-terminal "capping" is essential, and our studies on the RNA are aimed at answering some of these questions.

We are aware that the use of avian myeloblastosis virus for a model system is subject to criticism since it is not from a pure cloned viral stock, and contains a defective virus and two closely related leukemogenic viruses A and B present in varying amounts in various preparations from infected chicks (51). In spite of the presence of several closely related viruses in AMV, they *all* have the same primary structure at the 3' end just inside the poly(A) tail, in evidence from our laboratories (D. Schwartz, unpublished data). Many avian viral proteins have species and group specific determinants and are antigenically related so that the overall mechanisms of synthesis of at least most of the proteins should be similar in a mix of the two leukemogenic viruses for these initial studies. The amount of 70S RNA required for optimal translation is currently 8 μg/50 μl assay. This is much higher than that required for globin mRNA, for example, and represents a very large amount of virus. At present the availability of large amounts of AMV far outweighs the disadvantages of using a mixed virus.

4. Attempts to detect the presence of newly formed reverse transcriptase by its enzymatic activity using synthetic primers and templates

Some immunologists raise notes of caution about acceptance of antibody precipitates as conclusive evidence of the synthesis of viral message specified proteins, particularly in cell-free systems. Our choice of seeking the enzymatic activity of the reverse

transcriptase, which is more specifically associated with the viral message than any other protein one can at present think of, makes this type of viral translation assay a better test of message specificity than the presence of antigen-antibody precipitate proteins.

Preliminary experiments using synthetic templates, primers, and $^3$H-deoxynucleoside triphosphate precursors for the enzyme assay have given variable results in the wheat embryo system. Since the enzyme is capable of using many 3'-OH terminal oligonucleotides as primers it has been found to be necessary to prefractionate the cell-free protein synthesizing system prior to assay for the reverse transcriptase. Micro-phospho cellulose columns are currently being used to isolate the putative enzyme free of other inhibitory or stimulatory factors present in the wheat embryo system. These experiments are in progress.

5. Discussion

Our current experiments have been carried out in conjunction with chemical investigation of the primary structure of the RNA. We have shown earlier that AMV terminate in adenosine at the 3' end (2) and that most 35S RNA contains poly(A), all of which is at the 3' end (3). Oligonucleotide sequencing methods being perfected and used by Drs. Weith and Schwartz in our laboratories 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. They have recently (February, 1977) determined the primary structure of the region immediately adjacent to the 3' terminal-poly(A) sequence of the 35S RNA of RSV. A sequence of 20 nucleotide residues has been determined in RSV. The methods and details have been discussed in Section II.A; see also references 1 and 4.

Because of the exciting findings of a reiterated terminal sequence in the 35S RNA of RSV described above, part of our current proposal is aimed at studies with a synthetic triadecamer deoxynucleotide, complimentary to part of this reiterated sequence. It is proposed that such an oligodeoxynucleotide might inhibit oncogenic viral replication by acting as a competitor to the same sequence. As outlined in the proposal some of these studies involve its effect upon translation as well as transcription or mRNA processing and these protein synthesis studies carried out during the past year have placed us in an excellent position to interdigitate such studies with this ongoing research (see PROPOSAL).

## LEGENDS AND COMMENTS TO FIGURES AND TABLES OF OUR WORK

Figure 3 - This is a typical enzyme concentration curve obtained with the wheat embryo extract in response to heated 70S AMV RNA.

Figure 4 - This is a photograph of a Coomassie Brilliant Blue stained 7 - 18 per cent polyacrylamide, 0.1% SDS slab gel. The bands contain the following:

Band 1 and 5 - wheat embryo extract
Band 2 - phosphorylase A
Band 3 - protein mix: albumin, ovalbumin, chymotrypsinogen, and cytochrome C - the molecular weights are indicated at the left side of the gel
Band 4 - lysed avian myeloblastosis virus

Figure 5 - Comparison of stained gels with radioautographs made from the gels:

A - gels stained for proteins with Coomassie Brilliant Blue
B - three day radioautograph of the same gel. This gel which is not a very even one was chosen because it contains aliquots of the samples of incubation mix from an experiment using the pooled RNA samples indicated in Figure 7. Approximately 35 ul of the wheat embryo incubation mix were applied to the gel and 10 ul were counted as indicated in Table I

Figure 6 A - Radioautograph of gel containing $C^{14}$-AMV control proteins and proteins isolated in immune complex of the labeled AMV plus anti-AMV serum

This experiment indicates that when the lysed AMV was incubated with anti AMV serum overnight to form an immune complex, the AMV proteins appeared in the complex with no new bands appearing. Most of the AMV protein was present in the complex. The control AMV proteins are in bands 7 and 9 and the washed immune complex is in bands 2 and 5. The rest of the gel contains standard proteins and unlabeled AMV which are visualized on the stained gel (gel not shown)

Figure 6 B - Counts from $C^{14}$-AMV appearing in washed immune complex made with increasing concentrations of anti-AMV serum in the direct immunoprecipitation reaction

At 100 ul anti-AMV serum about 85 per cent of the counts in AMV proteins (761 cpm in 15 ug protein by Folin reaction) are present in the complex. At 200 ul anti-AMV all the counts are in the complex. In our experiments we have been adding about 10 ug of "cold" AMV to 100 ul of anti-AMV serum per reaction mix in order to be sure to precipitate all of the nascent viral specific proteins formed

Figure 7 - Isolation of various fractions of AMV RNA

This is a typical preparation of AMV RNA indicating the 70S RNA pool in gradient 1 which has been used in many incorporation experiments after heating to break up the complex. Gradients 2 and 3 indicate the type of 35S RNA we obtain from the pooled 70S RNA. Two concentrations of the same RNA are shown. A better fractionation may be obtained if small amounts of RNA are loaded on the tubes however for recovery purposes the larger amount is loaded on several gradients. The pooled fractions indicated were used in the experiments shown in Figures 5, 8 and 9 and in Table I A and B.

Figure 8 - Effect of different pooled fractions of RNA from the gradients indicated in Figure 7 on the incorporation of $S^{35}$-methionine into proteins using the wheat embryo system. We have arbitrarily indicated these pools as 4S RNA area, 10S RNA area, 18S - 28S RNA area, 35S RNA peak and over 35S RNA area. Aliquots of total AMV RNA and heated 70S RNA were also assayed.

Table I A - Incorporation of $S^{35}$-methionine into proteins using the wheat embryo system and various fractions of AMV RNA as indicated in Figure 7

Gels made from aliquots of these fractions indicate that (1) the smaller RNAs (except the 4S RNA area) make smaller proteins - see Figure 5 B. (2) proteins appear in the immune complex made against anti-AMV. Small RNAs apparently can make viral specific proteins in this cell free system

Table I B - This is a separate experiment where the various RNA fractions were separated into poly(A)+ and poly(A)- fractions using oligo(dt)-cellulose columns. All fractions except the 4S RNA area pool have activity. There is slightly more activity in the poly(A)+ fractions except in the 10S RNA area pool which had very little poly(A)+ in it and consequently we can't be sure of the single enzyme assay on that fraction. All other assays were carried out in duplicate. A preliminary gel indicates that the proteins made in the poly(A)+ and poly(A)- fractions from the same pool appear to be different, or if they prove to be the same they are made in very different ratios.

From Shapiro and August (7)

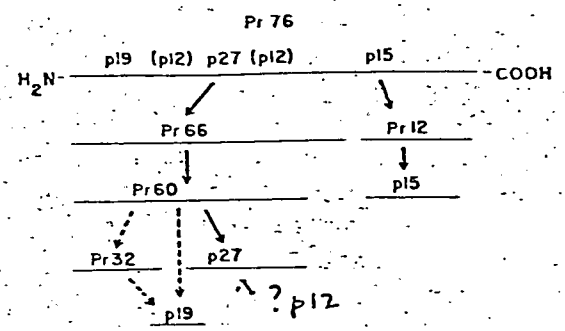



Fig. 1. Pactamycin map and cleavage scheme of avian oncornavirus precursor protein Pr76 (adapted from Vogt *et al.* [96].

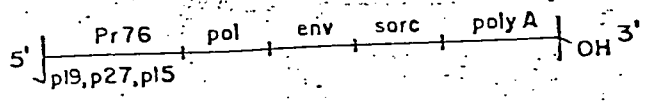



Fig. 2. Gene order of avian sarcoma virus.

Figure 2

ENZYME CONCENTRATION CURVE

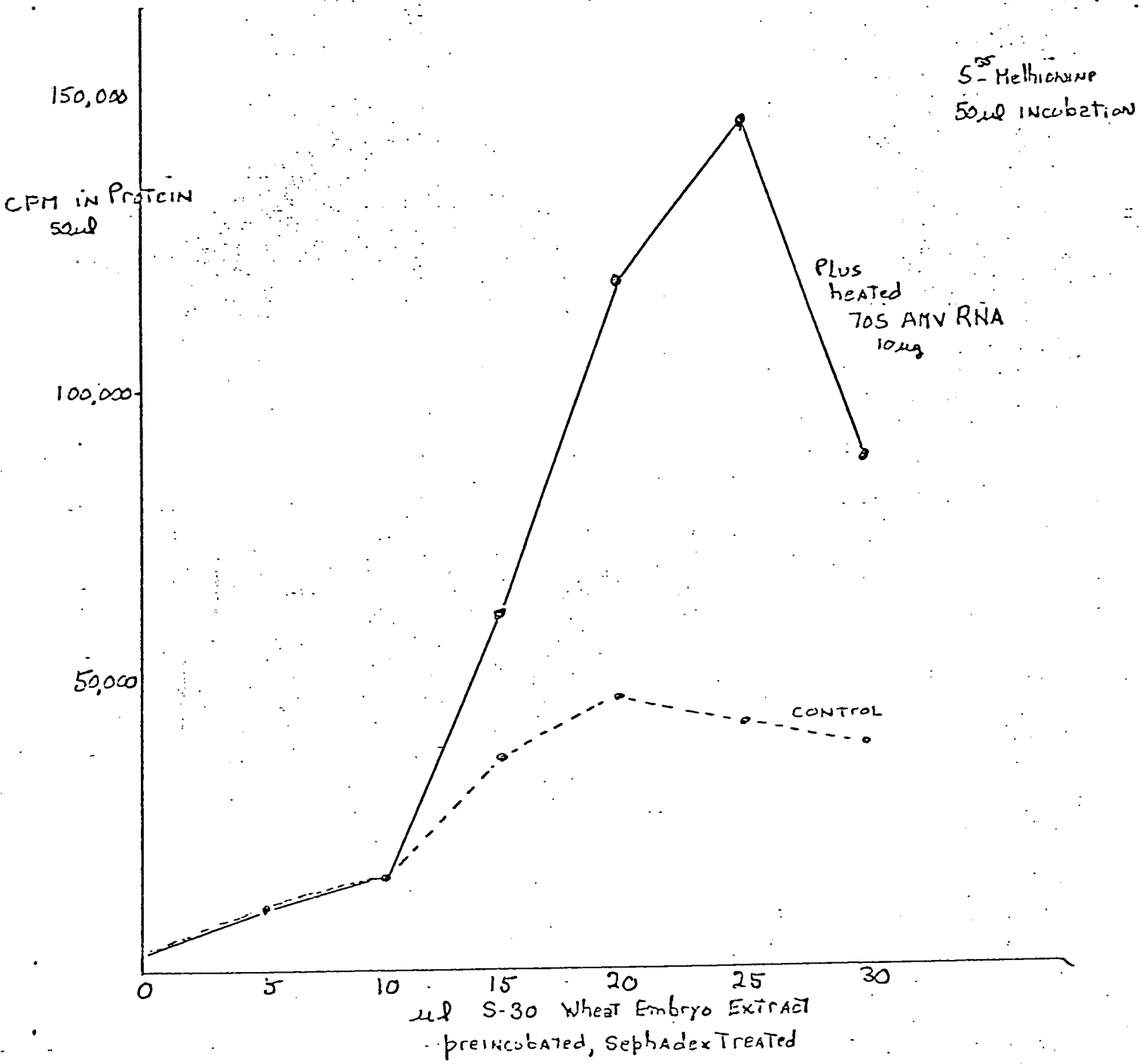



Figure 3

Coomassie Blue Stained 7 to 18 Per Cent Polyacrylamide SDS Slab Gel

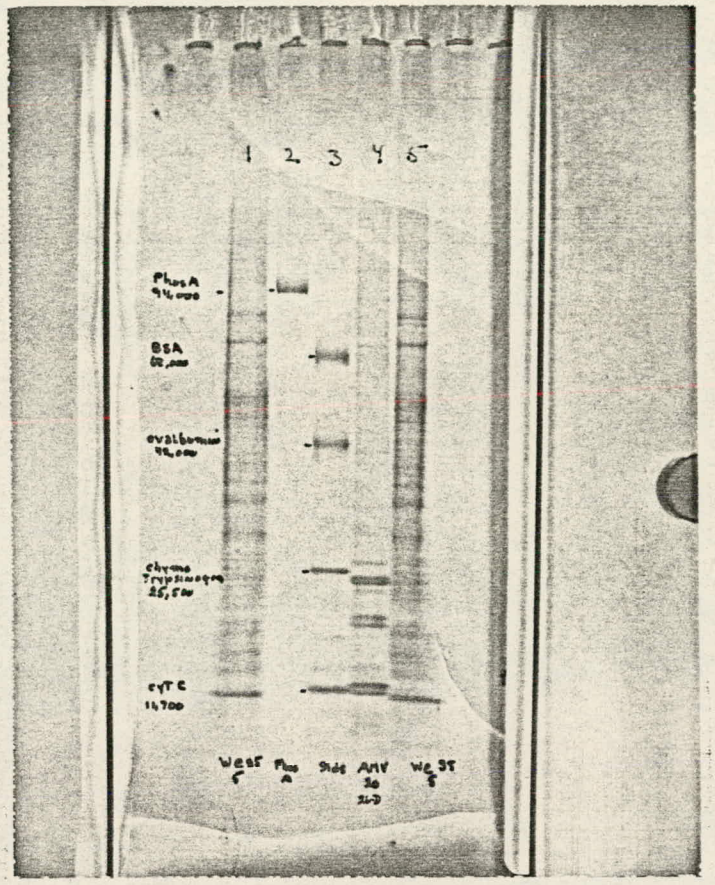
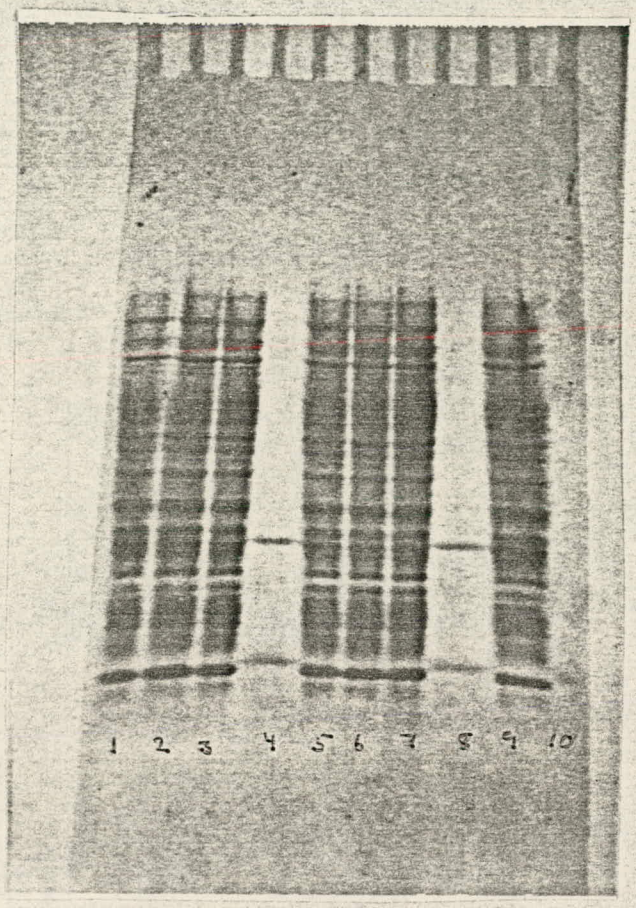


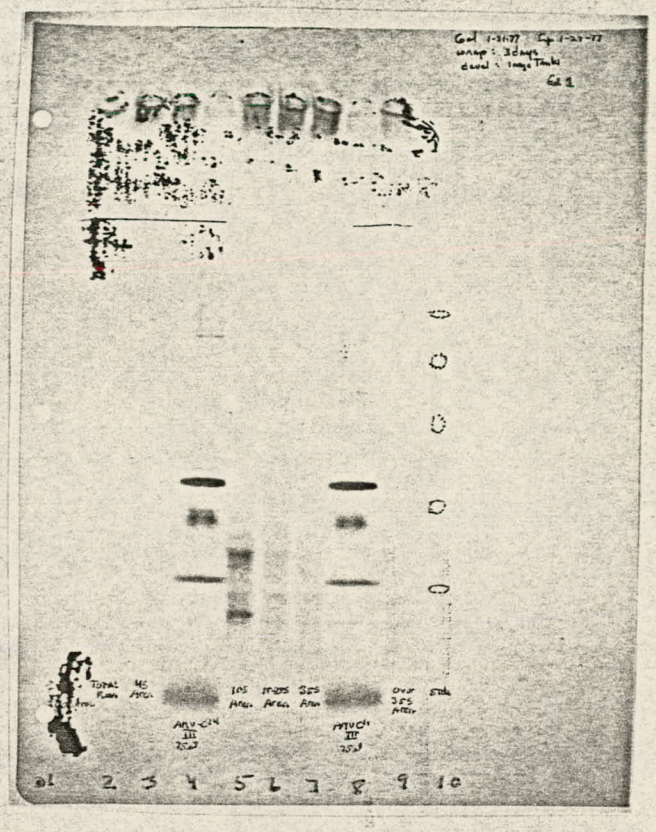

FIGURE 4

Comparison of Stained Gel with Radioautograph of Gel

5 A Stained Gel




5 B Radioautograph (14 days)




1 - Control
2 - Total RNA
3 - 4 S RNA area
4 - $C^{14}$-AMV proteins
5 - 10 S RNA area
6 - 18 - 28 S RNA area
7 - 35 S RNA area
8 - $C^{14}$-AMV proteins
9 - over 35 S RNA area
19 - Standard protein mix

FIGURE 5 A and B

Radioautograph of Gel containing $C^{14}$-AMV control proteins and proteins isolated in immune complex of $C^{14}$-AMV plus anti-AMV proteins

Bands 7 and 9 - Control AMV proteins
Bands 2 and 5 - Labeled proteins in immune complex

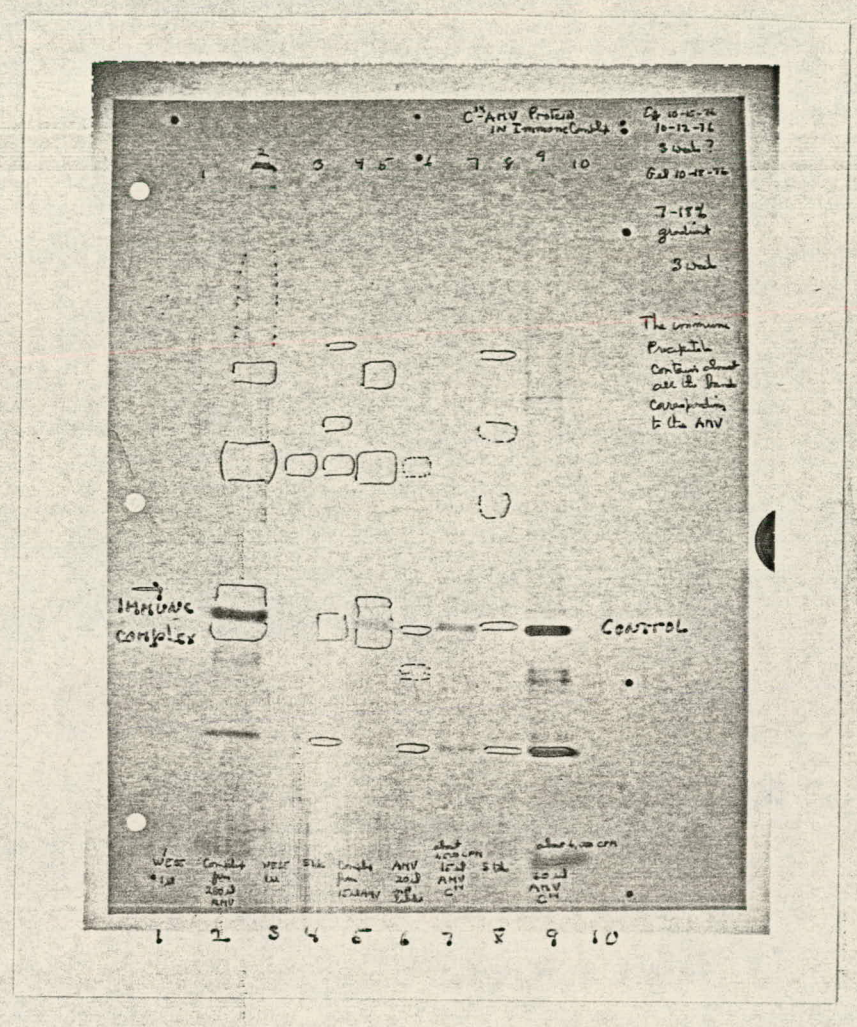



FIGURE 6 A

COUNTS FROM $C^{14}$-AMV FOUND IN IMMUNE COMPLEX WITH VARYING AMOUNTS OF ANTI-AMV SERUM

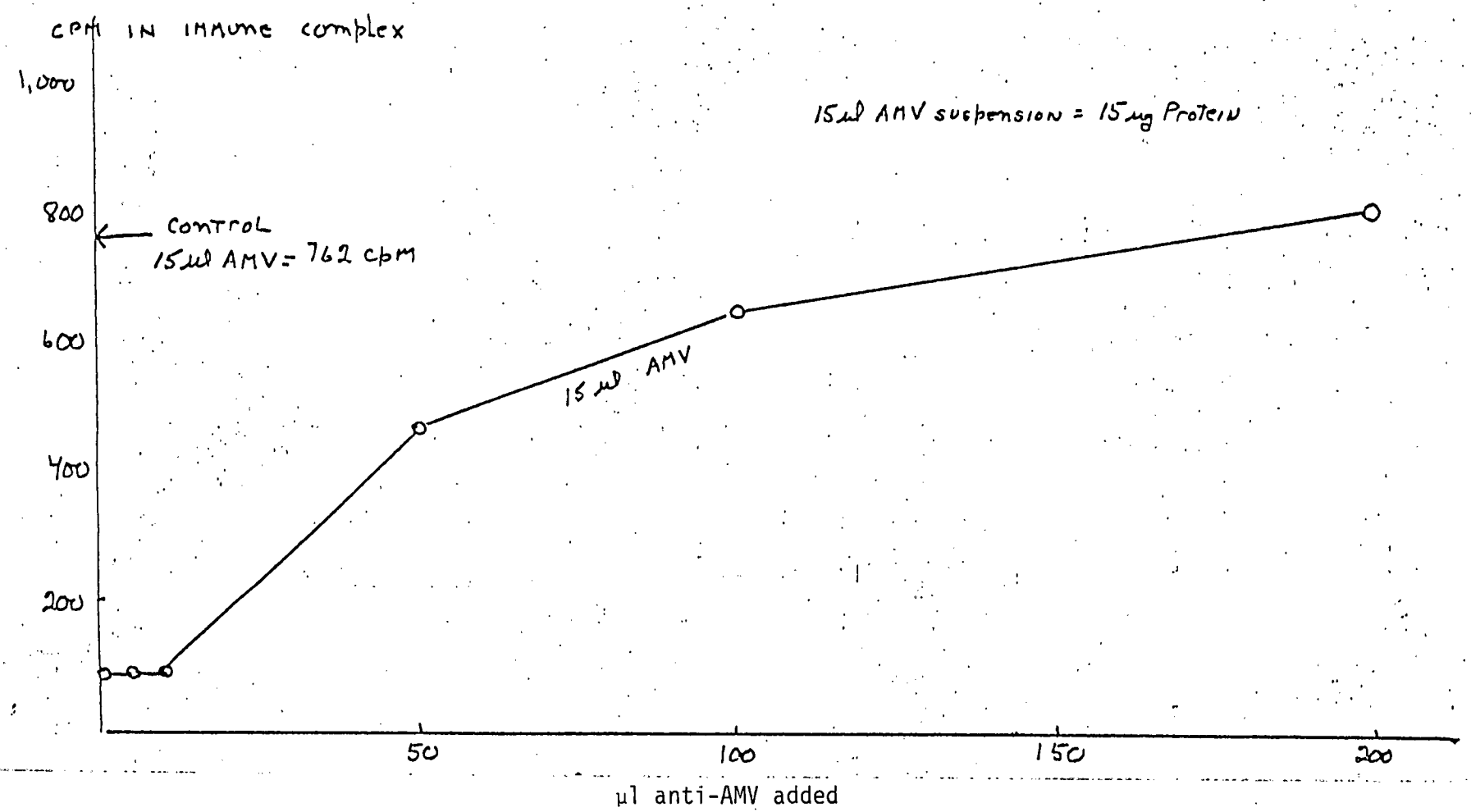
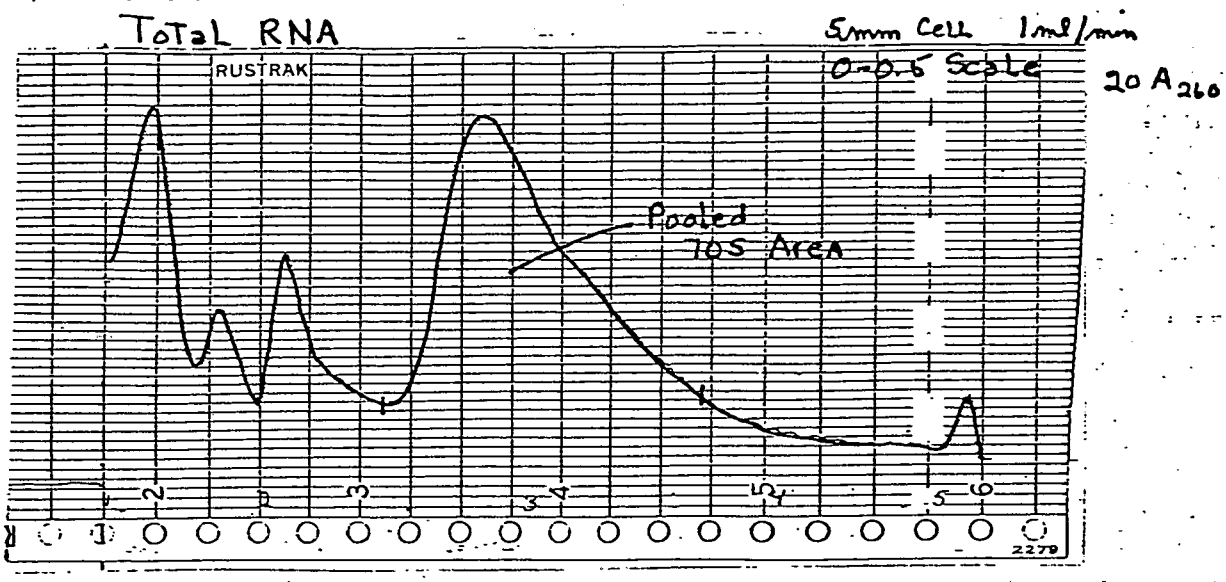
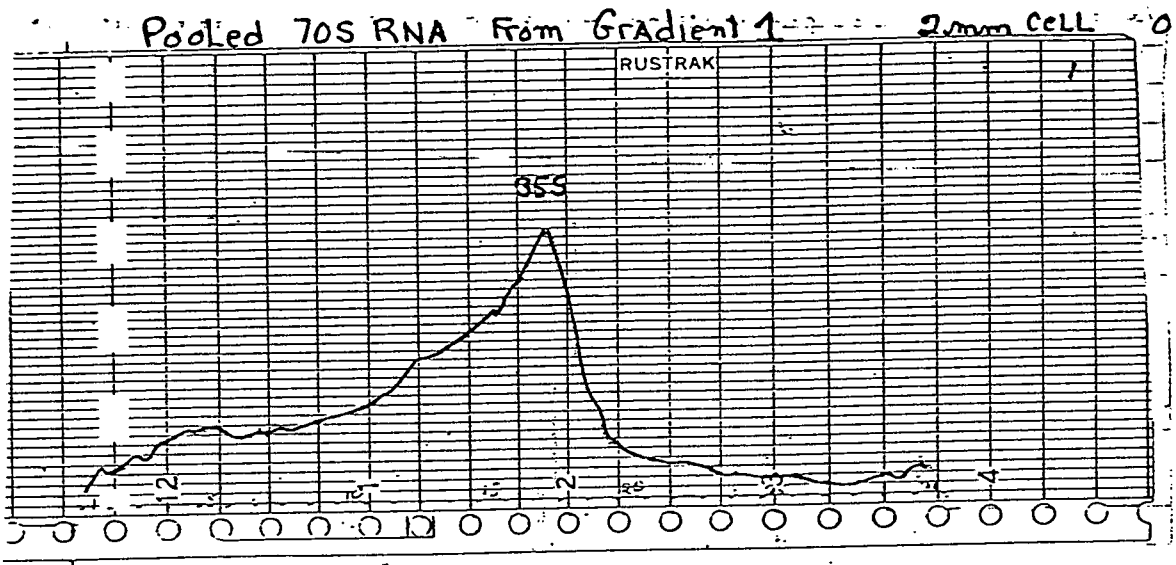


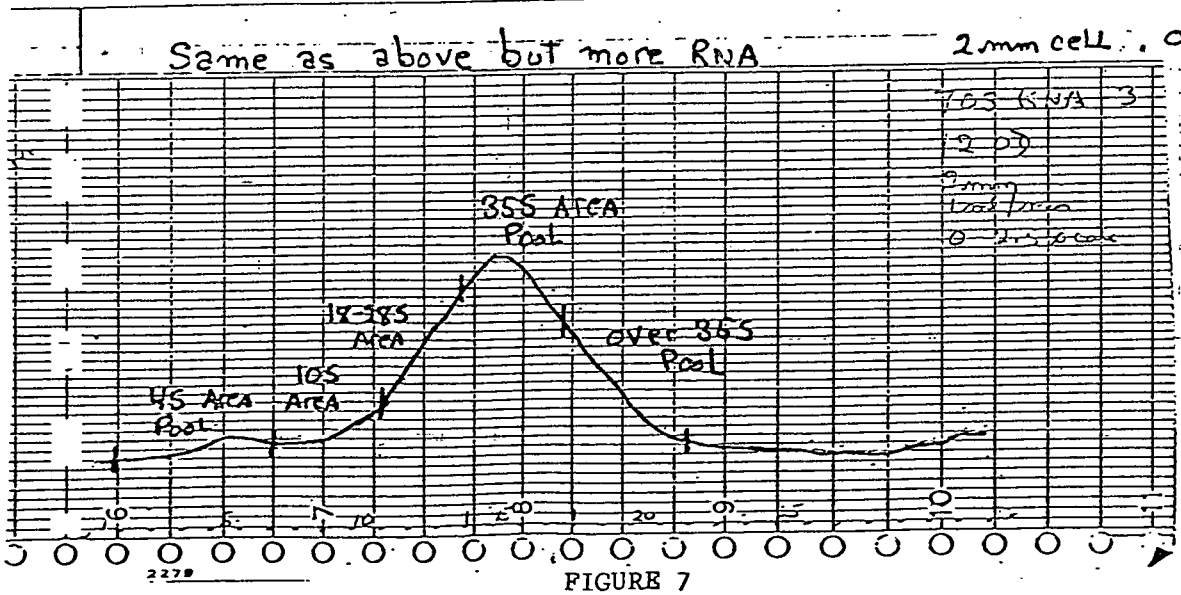

Figure 6B

Linear 5-30 % Sucrose, 0.2 % sarkosyl Gradient
Beckman SW 40 Rotor, 2.5 hours 5°C at 40,000 rpm




85 % DMSO 0 - 20 % Linear Sucrose Gradients
45 hours at 40,000 rpm




RNA pooled From gradients containing 12.0 $A_{260}$ units

FIGURE 7

STIMULATION OF INCORPORATION OF $S^{35}$-METHIONINE INTO PROTEIN OF WHEAT EMBRYO EXTRACT BY AMV RNA FROM GRADIENT SHOWN IN FIGURE 7

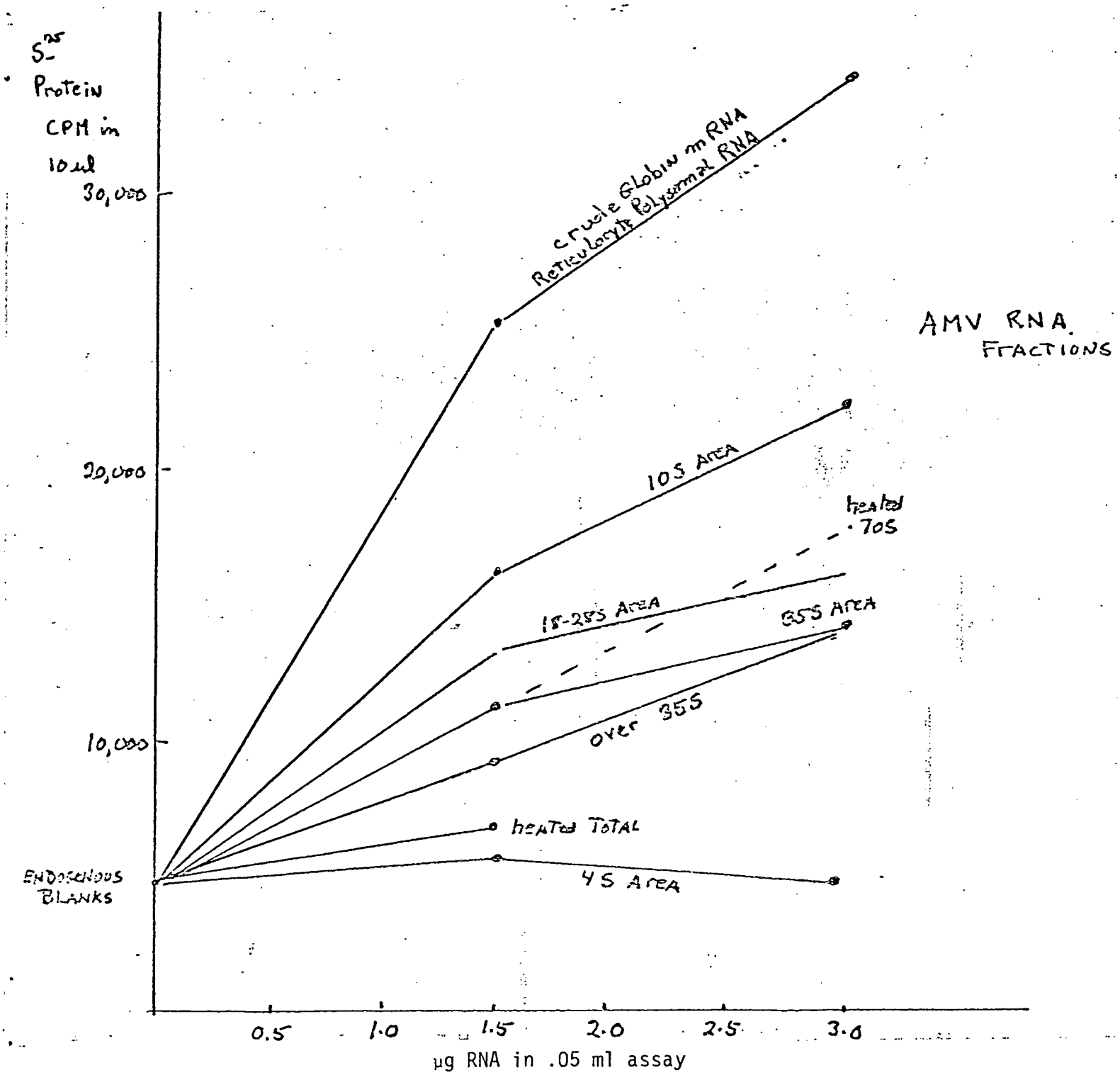



Figure 8

TABLE I A

Incorporation of $^{35}$S-methionine into proteins using the wheat embryo system and various fractions of AMV RNA from gradient centrifugations

| | CPM | CPM minus control | Stimulation |
|---|---|---|---|
| Endogenous control | 4346, 5165 } 4755 | - | |
| Reticulocyte polysome RNA (globin mRNA) | 24099 | 19344 | 5.07 |
| Total AMV RNA-heated | 7924 | 3169 | 1.66 |
| 70 S Pool-heated | 15554, 20950 } 18252 | 13497 | 3.84 |
| Samples from 70S RNA DMSO Sucrose gradient | | | |
| 35S RNA | 16007, 15612 } 15800 | 11405 | 3.32 |
| 4S RNA area | 5837 | 1082 | 1.23 |
| 10S area | 17334 | 12589 | 3.65 |
| 18 - 28S RNA area | 16007 | 11252 | 3.37 |
| 35S area | 15800 | 11405 | 3.32 |
| over 35S RNA area | 9839 | 5104 | 2.07 |

Each tube contained 4 ug RNA in 50 ul incubation, 10ul counted
45 mM KCl, 1.3 mM effective $Mg(Ac)_2$, .08 mM spermine, .02M HEPES, pH 7.5

TABLE I B

Pooled RNA fractions separated into poly(A)+ and poly(A)− RNAs

| | CPM | CPM minus control | per cent CPM | Stimulation | per cent RNA | % counts / % RNA | |
|---|---|---|---|---|---|---|---|
| | | | | | | poly(A)− | poly(A)+ |
| Endogenous 0° - 1 hour | 1268 | | | | | | |
| 30° - 1 hour ave of 4 | 2816 | | | | | | |
| 70S - unfractionated (all ave of 2) | 6189 | 3373 | | 2.19 | | | |
| poly(A)− | 5598 | 2782 | 48.7 | 1.99 | 54.8 | 0.89 | 1.13 |
| poly(A)+ | 5741 | 2925 | 51.2 | 1.88 | 45.2 | | |
| | | 5707 | | | | | |
| 35S - unfractionated | 4797 | 1981 | | 1.70 | | | |
| poly(A)− | 5357 | 2541 | 43.4 | 1.90 | 51.1 | 0.85 | 1.16 |
| poly(A)+ | 6125 | 3309 | 56.6 | 2.18 | 48.9 | | |
| | | 5850 | | | | | |
| 18S-28S area poly(A)− | 6253 | 3473 | 32.0 | 2.22 | 54.4 | 0.59 | 1.49 |
| poly(A)+ | 10207 | 7391 | 68.0 | 3.62 | 45.6 | | |
| | | 10864 | | | | | |
| 10S RNA area poly(A)− | 6489 | 3673 | 59.8 | 2.30 | 82.1± | 0.73 | (2.2) |
| poly(A)+ * (1 sample) | 5286 | 2470 | 40.2 | 1.88 | 17.9± | | |
| | | 6143 | | | | | |
| 4S RNA area poly(A)− | 3800 | 984 | (100) | 1.35 | 95.0 | | |
| poly(A)+ (no RNA) | -- | -- | | | 5.0 | | |
| Total RNA poly(A)− | 4042 | 1226 | 21.1 | 1.43 | 65.7 | 0.32 | 2.30 |
| poly(A)+ | 7408 | 4592 | 78.9 | 2.63 | 34.3 | | |

* The accuracy of the 10S RNA poly(A)+ may be poor because of low $A_{260}$
All tubes contained 2 ug RNA in 50 ul incubation, 10 ul counted

C. The Relationship of Diadenosine Tetraphosphate ($Ap_4A$) to the Rapidity of Cell Growth - P. C. Zamecnik

The first step in protein synthesis was discovered in these laboratories in 1955 (52,53) under the aegis of an ancestor of our present contract, and may be depicted as follows:

$$(1)\quad pppA + aa_1 + Eaa_1 \rightleftarrows aa_1\text{-}pA^{\cdot}Eaa_1 + pp$$

In 1966, while examining this reaction in the cell of an optical rotatory dispersion apparatus, we observed the appearance of an unusual Cotton effect centered around 267 mμ, which increased in magnitude with time of incubation of the participants of reaction (1) above (54,55). It was absent when the enzyme was omitted; and its development was dependent on the presence of all three components of the left half of equation (1) above. We found that the explanation was as given in equation (2) herewith:

$$(2)\quad aa_1\text{-}pA^{\cdot}Eaa_1 + pppA \rightleftarrows A(5')pppp(5')A + aa_1 + Eaa_1$$

In other words, the ATP (pppA) was reacting both in the forward part of equation (1), in which $aa_1$-$pA^{\cdot}Eaa_1$ and pp are formed; and in the back reaction as well, in competition with pp. $Ap_4A$ was so readily formed in the <u>in vitro</u> enzymatic system that we suspected it might also be formed <u>in vivo</u>. We therefore looked carefully for its presence in rat liver slices, mouse ascites tumor cells, and E. coli, and with some difficulty found $Ap_4A$ in each of these living cell types (56,57). We also found in enzymatic <u>in vitro</u> reactions that a number of other pyrophosphate containing compounds, which we may designate as ppN or pppN (N = nucleoside, deoxynucleoside), are able to substitute for inorganic pyrophosphate in this reaction (57) as described cryptically as follows:

$$(3)\quad a)\ aa_1\text{-}pA^{\cdot}Eaa_1 + ppN \rightleftarrows NpppA + aa_1 + Eaa_1$$

$$b)\ aa_1\text{-}pA^{\cdot}Eaa_1 + pppN \rightleftarrows NppppA + aa_1 + Eaa_1$$

Certain pyrophosphate analogues such as pcp and pnp and their nucleotide derivatives will also participate in the type of reaction shown in equation (3).

We have taken up the study of the possible metabolic role of $Ap_4A$ and related compounds during the past year, having greater facility at present with separation of $Ap_4A$ from other nucleotides, and with the help of precursors of higher specific activity, plus the use of tissue cultures of mammalian cells.

Our initial observations were that in contrast to the constancy of ATP levels in a variety of rapidly and slowly growing cells, the levels of $Ap_4A$ varied over a 100-fold range, being around 1-3 x $10^{-6}$M in rapidly growing cells in culture and around 1 x $10^{-8}$M in slowly growing cells (58). Furthermore, the levels of $Ap_4A$ declined to the $10^{-8}$M range when the growth rate of the rapidly growing lines was slowed down by serum deprivation, selective amino acid deprivation, or hydroxyurea addition. The $Ap_4A$ levels in whole animal livers and tumors were very succeptible to anoxia, more so than the levels of ATP.

These and other observations are described in detail in a recent publication of ours in the PNAS (58), and therefore will not be duplicated in the present report. In contrast to cyclic AMP and ppGpp, whose levels rise in general under

growth restrictive conditions, $Ap_4A$ appears to be related to the growth process in a positive way--i.e., its levels are higher where growth is most rapid, and its levels decline when the growth process decelerates.

Reprints Removed

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## III. Publications During the Past Year (1976-1977)

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