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

PROGRESS REPORT

by

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Summary of Proposed Technical Scope of Research

The laboratories are engaged in biochemical studies of the high molecular weight RNA of an oncogenic virus (AMV), which causes nucleic acid and protein synthesis to be continuously turned on in the myeloid cells of the chick. We are also investigating the mechanism by which an aminoacyl-tRNA synthetase recognizes its own cognate tRNA, in one of the important unsolved mysteries of molecular biology, which is central to achieving a correct translation of the message of the gene into the functional operation of the cell.

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I. Introduction and scope of project

The enclosed reports represent an interwoven effort by members of the Huntington Laboratories to study the mechanisms of normal protein synthesis and their alteration in the presence of RNA oncogenic viruses. Greater understanding of the details of the transfer of genetic information leading to synthesis of specific proteins albeit viral or normal will hopefully aid in our understanding of the effects of radiation on living cells.

Last year we were not asked to submit a complete report and proposal because the date of initiation of the contract year was shifted from September 1 back to July 1 and the report period did not cover an entire year. We did submit a very brief report but for the sake of clarity and continuity some of last year's material will be mentioned in this report. Also, copies of publications submitted during that period are included and referred to. Some parts of this progress report are included in the accompanying proposal in order to clarify the latter.

For several years we have been focusing on the early stages of protein synthesis where an aminoacyl tRNA ligase recognizes its cognate RNA. It is in this recognition area where growth regulation and specificity can be incurred readily, for if the enzyme fails to recognize its RNA, the normal regulated protein metabolism may be altered. Evidence has accumulated that primary, secondary and tertiary structures of tRNA all play roles in determining its ability and selectivity in associating with the correct aminoacyl ligase. This recognition area is being scrutinized in normal systems using two approaches. First is the isolation of mutant tRNAs or aminoacyl tRNA synthetases with the aim of altering the rate and amount of proteins synthesized. It should be possible to pinpoint recognition sites by this approach. The second line of attack is to alter purified aminoacyl ligase or tRNA chemically in vitro and to study the changes in the recognition reaction.

Other interrelated biochemical studies are focused on the mechanisms by which an oncogenic virus (AMV is used as a convenient model) modifies and subverts the normal cell protein metabolism. These involve the characterization of the viral RNA. The finding of the presence of tRNA in RNA oncogenic viruses stimulated us to determine whether the minor base patterns of the viral tRNA differed significantly from those of the host cells, using the minor base method mentioned earlier. These were described last year. This year we have investigated the amino acid acceptance patterns of the viral tRNAs, both tRNAs free in the virion and those bound in the 70S RNA complex.

A second program was initiated two years ago directed at the high molecular weight RNA of the virion. The mechanism of the transfer of the genetic information of the virion to the host cell has many analogies to the effect of bacteriophage infection upon normal host bacteria. It is hoped that similar insight into the regulation of mammalian protein synthesis may come from a study of the mammalian virus with normal cells. Since little has been known about the chemistry of these viral RNAs, we have been isolating and purifying various RNA fractions and identifying the 3'-OH end segment of the molecule as poly(A) of both the 70S RNA and the 35S RNA derived from the 70S material. Procedures aimed at improving current sequencing techniques of high molecular weight RNAs have also been explored.

II. Specific reports

A. Investigation of the mechanisms by which an RNA oncogenic virus subverts the normal patterns of growth regulation

1. Characterization of the viral nucleic acids

A. Introduction

The projects being carried out on the RNA's of the avian myeloblastosis virus are mainly involved with the chemical characterization of the nucleotide structure of the specific viral RNA's. Some effort has and continues to be placed in the problem of obtaining μ g quantities of viral high molecular weight RNA in undegraded condition. The bulk of the current studies are focused on the high molecular weight RNA, the "35S" RNA of AMV which has been implicated in several biological functions, i.e. polymerase template and mRNA activities.

During the past year we have extended our previous observations that the 3'-OH terminus of the high molecular weight RNA's of the Avian Myeloblastosis Virus is an adenosine residue (1). We have found that this 3' end of the 35S RNA contains a polyadenylic acid sequence of at least 30 nucleotides (2). Furthermore, this terminal poly(A) sequence is the only poly(A) stretch we have found in the approximately 6000 residue 35S molecule (or closely related family of molecules). There are other reports of oncogenic viral RNA's containing runs of poly(A) (3-6), but to our knowledge this is the first report which identifies a poly(A) sequence at the 3'-terminus, and is in addition the only poly(A) sequence in the entire molecule.

We are currently trying to determine the nucleotide sequences just internal to this poly(A) sequence. This is the region of the RNA which may be involved in the attachment to the reverse transcriptase or for enzymatic activities in relation to its activity as mRNA. We are presently isolating poly(A) containing fragments with specific enzymes hoping to find short terminal segments containing other nucleotides in addition to adenylic acid that can subsequently be sequenced by other methods. A smaller portion of our efforts are also involved in investigating more promising nucleotide sequencing methods than are available for this type of large RNA. For these studies model oligonucleotides are presently being used in a variety of chemical reactions described later.

B. Characterization of the High Molecular Weight RNA of AMV

1. Introduction - The findings discussed in this introduction are well documented in the accompanying proposal

The high molecular weight RNA has been shown to be implicated in several biological functions. For example, the viral reverse transcriptase is capable of synthesizing DNA using the high molecular weight viral RNA as template, and the DNA from cells infected with AMV contains sequences complementary to the viral RNA. In this way virus specific mRNA can be transcribed from infected cell DNA. In this system the large viral RNA is the template and there is evidence that a lower molecular weight fraction of RNA, possibly tRNA, may act as primer for the transcriptase.

The high molecular weight RNA has also been demonstrated to serve as messenger RNA in several cell free E. coli systems which are capable of synthesizing proteins identified as being viral specific.

It has been demonstrated by a number of groups that the virus RNA from Rauscher, Avian Myeloblastosis, Feline leukemia, Moloney Sarcoma, Rous Sarcoma, visna, Mason-Pfizer agent, reo, Eastern Equine Encephalitis, polio, and Sindbis contain sequences of polyadenylic acid. Others have shown that mRNA, both viral and non-viral, contains poly(A) segments and evidence has been presented that the poly(A) of some of these mRNAs contains the 3'-OH terminus of the molecule. In the cases of the RNAs from poliovirus and reovirus there is evidence that the poly(A) segments also contain the 3'-termini. Until the present report, however, there has been no definitive evidence that high molecular weight oncogenic viral RNA terminated in a 3'-OH poly(A) sequence, although it seemed reasonable, by extension, that the poly(A) segment of the RNA released from AMV viral particles might also contain the 3'-OH end of the molecule. Runs of poly(A) at or near the 3'-OH terminal of the high molecular weight viral RNA may be necessary for attachment to the transcriptase (RNA:DNA polymerase) or for enzymatic activities in relation to its function as messenger RNA. However, the function or the necessity of these residues in viral RNA is not clear at present. These poly(A) sequences and the mechanism of their addition may, however, be critically involved in the activation of latent viruses. For example, they may be sites of initiation of transcription of DNA or somehow involved in transport of viral material out of the nucleus where it is transcribed.

Last year we identified adenosine as the predominant terminal 3'-OH residue of the 35s RNA from AMV. This finding made it reasonable to investigate the 3' end of the molecule further to see whether it terminated in a poly(A) residue. See enclosed reprints of references 1 and 2.

On the other hand, the RNAs of many plant and bacterial viruses show a degree of similarity at the 3'-OH terminus of the molecule and it is believed that the 3'-termini are important in binding the viral RNA polymerase. These RNAs have been found to terminate in -CCA. A fascinating report is that of Chapeville et al., that the terminal 3'-OH sequence of the Turnip Yellow Mosaic Virus serves as the site for esterification of valine. In essence, it would seem that the 3'-OH end of that large molecular weight viral RNA is tRNA_{val}. The function of such a terminus is not known. Since Chapeville's finding, several other plant and possibly some bacterial viral RNAs have been found which may terminate in tRNAs. This is discussed in the proposal.

2. Results

Because of the possibility of the 35s RNA terminating in a run of poly(A) or an equally intriguing possibility of its terminating in -CCA capable of bearing an amino acid mentioned above, last year we initiated studies to identify the penultimate nucleotide of the RNA since we had shown the 3'-OH terminus to be adenosine (1).

a. Studies on the penultimate nucleotides of AMV and TMV

Several laboratories (7-14) have successfully carried out several cycles of stepwise degradation of high molecular weight RNA, for example, TMV RNA (11). These investigations employing plentiful supplies of RNA are able to identify the released nucleoside following chromatography by optical methods or the biological system may be incubated *in vivo* using labeled RNA precursors, followed by isolation of RNA sufficiently "radioactive" that the released nucleoside derivative can then be counted and quantitated following chromatography.

We chose to use a combination of two methods: 1) the borohydride method currently used to identify the 3'-OH terminal nucleoside (1) and 2) the stepwise degradation of the RNA by base catalysis following periodate oxidation of the RNA (11). This type of analysis has been used by Kamen, (8) on P17 RNA. The method is inefficient since we sacrifice the aliquot of RNA which is treated with borohydride for identification of the 3'-OH terminus, but on the other hand it enables us to get termini that are "hot" enough to quantitate. We have carried out such an experiment on a control sample of TMV RNA. This has a known terminal sequence and is also a high molecular weight RNA.

The method used by us works for this type of RNA. A comparison of the results of Steinschneider and Fraenkel-Conrat (11) using the base catalyzed degradation of large amounts of RNA with our results using the combined method and micro amounts of TMV RNA was as follows: the terminal 3'-OH nucleoside was 87.6 and 87.7% adenosine in their experiment and 90.7, 91.4, and 90.0% adenosine by the borohydride labeling technique in our hands. The penultimate nucleoside was 57.8 and 67.8% cytidine by their base catalysis method while using the borohydride technique we found 73.1% cytidine. This combined procedure enabling us to use μ g amounts of RNA is successful for such TMV RNA, but we have not had such good results with three 35S RNA preparations from AMV so far tested. We find that the phosphatase treated control which had no effect on the 3'-OH end of TMV RNA, uncovered varying amount of uridine and cytidine from the viral 35S RNA fraction. The experiment showed that the AMV 35S RNA pool had about 90% of its 3'-OH end terminating in adenosine but that there were also some molecules present ending in a 3'-phosphate. Subsequent isolation of the penultimate nucleotide indicated a spread of three nucleotides, so that we are unable to indicate which one is the penultimate nucleotide of the pure 35S RNA.

From these experiments we concluded that in order to carry out meaningful sequence studies on the 35S RNA it would be necessary to free the 35S RNA from closely related material terminating in a 3'-PO₄ since the sequencing procedure required a phosphomonoesterase step that subsequently exposed new 3'-OH termini which contaminated our results.

Slow but significant progress has been made in obtaining sharper peaks of 35S RNA using the DMSO-sucrose gradients described earlier in our last report. It should be pointed out that our isolation procedures are not carried out using tracer amounts of pulse labeled RNA but instead are designed to isolate μ g amounts of virus. Pulse labeling with high specific activity RNA precursors does yield sharp labeled 35S peaks in our hands, but these fail to show that the sharp radioactive peaks are mixed with varying amounts of degraded virus which did not label during the pulse and which are not visualized in the tiny amounts of labeled RNA isolated.

We felt at the time that further attempts to sequence the 35S by this method of sequential degradation would not be fruitful and that it might be more promising to investigate the possibility that the 35S RNA might have a poly(A) sequence at its 3'-OH terminal. Consequently we have put aside these experiments until we are able to obtain very pure fractions of 35S material and have concentrated on techniques which would demonstrate the presence of poly(A) segments on the 3'-OH termini of the virus RNA. It has already been shown by others that AMV RNA does contain segments of poly(A) (3), but it has never been demonstrated to our knowledge that these are at the 3'-OH end of the molecule.

b. Investigation of poly(A) sequences in the viral RNA

(1) Initial enzyme studies

Before we attempted to isolate poly A fragments from viral RNA we wanted to find out whether such a condition was a possibility, that poly(A) might be on the 3'-OH end of the molecule. In order to do this we used two enzymes, ribonuclease A and ribonuclease T1, which have specificities useful for cleaving the molecule in a manner to isolate runs of poly(A). We also made use of the fact that we could label the intact RNA at its 3'-OH end using the periodate- H^3 -borohydride method (1) prior to its being subjected to ribonuclease hydrolysis. Figure 1 indicates what would happen to terminally labeled RNA when hydrolysed in different ways. Our routine assay for labeled nucleoside trialcohols does not detect nucleotides or larger fragments since they remain at the origin of the two dimensional thin layer cellulose plates (15). We made use of this fact in the following experiment. The purpose of the experiment was to see whether either enzyme released a terminal labeled nucleoside trialcohol from the terminally labeled viral RNA. Figure 1 indicates the possible types of labeled fragments we might find following hydrolysis.

Tables I and II indicate the results using ribonuclease A and ribonuclease T1 respectively. When terminally labeled 35S RNA was subjected to Ribonuclease A digestion (Table I) there was very little labeled terminal nucleoside released compared with the total terminal nucleosides present as determined by piperidine hydrolysis. This indicates that only about 7 per cent of the ends terminated in pyrimidine-A and that 93% of the 35S RNA could terminate in -GA or -AA.

A separate experiment carried out on 70s RNA using ribonuclease T1 is indicated in Table II. In this case almost no terminal labeled adenosine was released compared with its control. This would indicate that less than 3% could have terminated in -GA.

We concluded from these two preliminary experiments that the viral RNA could indeed terminate in an A-A sequence at the 3'-OH end. This small bit of confirmatory evidence of a possible terminal poly(A) encouraged us to try to isolate poly A fragments from the high molecular weight RNA.

(2) Isolation of poly(A) from viral RNA

We have used a periodate- $[^3H]$ -borohydride labeling technique on both the total nucleosides of RNA hydrolysates and the 3'-OH terminus of the RNA in order to get ratios of the number and kind of total nucleosides compared with those in the 3'-OH terminus of the same molecule.

Much of the following material has been taken from our paper just published (2) and added to this report.

We have previously reported (1) that the 3'-OH end of the "35S" RNA released from the 70s RNA isolated from AMV contained in the plasma of infected chicks was largely adenosine. We here present evidence that all this adenosine is the 3'-terminus of a poly(A) segment and that all poly(A) segments isolated have a 3'-OH terminus.

Avian myeloblastosis virus (AMV) BAI Strain A was obtained through the courtesy of Dr. and Mrs. Joseph Beard via the Virus Cancer Program. Virus from fresh plasma (540 ml) of infected chicks was partially purified by differential centrifugation and the RNA was extracted by modifications of the method of Duesberg (16,17) as described by us earlier (1). This preparation yielded a total of 86 A_{260nm} units. Viral 70sRNA was isolated from 5-30% sucrose gradients using a Spinco SW 41 Rotor (1). The 70s material from 6 gradients was pooled and reprecipitated. The yield of RNA was 25 A_{260nm} units. The 70s RNA (21 A_{260nm}) was dissolved in 80% dimethyl sulfoxide (Me₂SO) containing 0.05 M Tris, pH 7.5, .005 M EDTA and applied to 80% Me₂SO, 0-20% sucrose gradients in order to isolate the "35S" material and free it of the 70 associated 4s RNA. The Spinco SW 41 Rotor was used at 40,000 rpm for 48 hours at 25°. External markers of 28, 18 and 4S RNA were used to estimate the S value of the viral RNA. All of the material was pooled except that traveling in the 4s region of the gradient. The pooled material did not give a sharp peak at 35S but consisted of a broad band ranging from about 10-35s. The pooled samples from four gradients containing 2 µl diethylpyrocarbonate per ml were made 1 M in LiCl by addition of 4M LiCl, and precipitated by the addition of 2.5 volumes of 95% ethanol. The RNA was dissolved and reprecipitated as before. Yield: 11 A_{260nm} units. For brevity the pooled RNA sample has been termed "35S" RNA, but it should be emphasized that all the RNA from the 70s material except that 4s material has been included, whether or not it is nicked "35S" RNA or other viral RNA, since Monier *et al.* (18) have reported that runs of poly(A) in AMV were found in the more slowly sedimenting areas of sucrose gradients. Poly(A) was isolated from the viral RNA fraction by the method of Mendecki *et al.* (19) with minor modifications. Both T1 ribonuclease, 140 units (Worthington) and ribonuclease A, 20 µg (Worthington) were used per ml incubation mixture. The cloudy suspension obtained by the SDS extraction of the RNA retained by the filter was centrifuged for 10 min at 12,000 x g, and the clear supernatant containing the poly(A) segments was removed. Carrier DNA free of RNA was added (200 µg) in order to help to co-precipitate nucleic acids which were precipitated by the addition of 2 volumes of alcohol. The poly(A):DNA precipitate was dissolved in 0.5 ml of 0.2 M NaCl and reprecipitated using 2.5 volumes alcohol. The final pellet was lyophilized to remove traces of alcohol and was then dissolved in 250 µl H₂O. A solution of DNA (200 µg in 250 µl H₂O) was carried through all subsequent operations as a "blank" control.

In order to determine the 3'-OH terminal nucleotide of the viral RNA we have used the periodate-oxidation -trinitrated borohydride reduction procedures perfected for microanalytical analysis by the Randeraths (20-22) following earlier procedures (10,7,23) which have more recently been used by others (24,25,8,26). The exposed cis-hydroxyl groups on the 3'-terminal nucleoside of

an RNA chain are oxidized to dialdehydes with periodate and subsequently reduced with tritium labeled borohydride, yielding an RNA containing labeled 3'-termini. Alkaline hydrolysis of the intact RNA yields the terminal nucleoside as a labeled trialcohol, thus identifying the terminal 3'-OH-residue of the chain. We have adapted this procedure for terminal labeling of the high molecular weight viral RNA (1). The 3'-OH terminus of intact RNA is labeled and the RNA is hydrolyzed by piperidine. The terminal labeled moiety is released as a labeled nucleoside trialcohol while the remaining hydrolysate consists of mononucleotides. The nucleoside trialcohols of adenosine, cytidine, guanosine, and uridine are isolated by thin layer chromatography on cellulose plates using cold carrier trialcohols while any nucleotides remain at the origin under the conditions used. By this procedure we have shown the major terminal 3'-OH of the "35s" RNA of AMV to be adenosine (1). For the current studies we have used the same conditions with the following modifications: DNA carrier was present in the RNA fraction, the periodate concentration was .005 M, and the phosphate concentration was reduced from 0.1 to 0.015 M following addition of the [^3H] borohydride (Amersham 6.4 Ci/mole).

The determination of the total base in the RNA utilized the same method of the Randeraths (20-22). In this case the RNA was hydrolyzed enzymatically to nucleosides prior to labeling by the periodate-borohydride reaction. We have used this method recently to determine the minor bases present in the 70s associated tRNA of AMV (27).

In order to estimate the 3'-PO₄ termini the RNA sample (ca. 2 A_{260nm} units) was incubated for 30 min at 37° with *E. coli* alkaline phosphatase, 2 µg, in 0.02 M ammonium acetate at pH 7.4, 0.001 M MgCl₂ in a total volume of 110 µl. This dephosphorylated sample was then treated as described above for the determination of 3'-OH termini and total base.

Figure 2 outlines the experimental plan by which the data given below were obtained.

Table III indicates the results of such an experiment, giving the radioactivity found in the total nucleoside and the radioactivity in the 3'-OH terminal nucleoside of a separate aliquot of the same sample. The per cent of radioactivity in each nucleoside is also indicated. The samples actually counted were the nucleoside trialcohols. Tables IV and V utilize the data from Table III in order to indicate some of the properties of the isolated poly(A) fragments. It should be pointed out that the low values marked with * in Table 3 are relatively unreliable due to low sample radioactivity relative to the blanks; however, the other samples have a counting error $\leq \pm 5\%$.

The "35s" pooled sample has adenosine as the predominant 3'-OH terminal nucleoside. This is in agreement with our previously published results (1) and those of Glitz (28).

With regard to the poly(A) segments, Table IV indicates that the poly(A) contains 0.65 per cent of the total "35s" nucleotides; however, 2.5 per cent of the adenosine in "35s" is found in the poly(A). Considering the differences in the techniques, this agrees moderately well with those found by

Gillespie *et al.* (3). The data in Table IV,3 indicate also that poly(A) is attached to the 3'-OH end of the molecule and that possibly all of the poly(A) in the "35s" RNA contains a 3'-OH terminus (V,IIC). To our knowledge this is the first definitive evidence of poly(A) segments at the 3'-OH terminus of an oncogenic viral high molecular weight RNA.

Table V gives an indication of the size of the RNA fractions by comparing the ratios of ends to total nucleotides. The original control (IA) has a calculated molecular weight of 1.75×10^6 ; however, following phosphatase treatment (IB), the calculated molecular weight dropped to about 775,000, indicating that the "35s" molecules as isolated had a few breaks. Since mainly U and C are liberated after treatment with phosphatase (III), the degradation of 35s is probably due to a ribonuclease of the same specificity as that of ribonuclease A. The broad peak isolated from the Me₂SO sucrose gradients had an apparent molecular weight more in the vicinity of 800,000 than 1.75×10^6 and agrees with the estimation of 1.25x as many phosphate termini as 3'-OH termini (V,IC). The poly(A) segments are about 30 nucleotides long (V,IIA,B). We also arrived at the same figure of 30 nucleotides in poly(A) using completely separate plates, and calculating via percentages of poly(A) in the original sample. This figure is in agreement with the small size for the poly(A) segments found in the reo and polio viruses (29,30). The poly(A) samples may contain small amounts of other nucleotides, as discussed by Horst *et al.* (31) for RSV RNA, but our data on this point are not reliable due to low counts in the RNA relative to the various blanks. Table VI represents a simplified version of the data from the other tables.

Since treatment of poly(A) with phosphatase did not change the end labeling (V,IIC), there were no terminal phosphate moieties and all the poly(A) isolated was on the 3' terminal end (Table IV). Therefore, not only is all the poly(A) in the 3' terminus, but as Table IV indicates, all of the terminal A of the "35S" RNA could be a part of a poly(A) segment.

Four separate experiments have been carried out in a fairly similar fashion. All have evidence of poly(A) segments terminating in a 3'-OH. The experiment described is our best one for quantitation. In two earlier experiments, one using 70s RNA and one using total RNA, the amount of poly(A) actually isolated was significant but enzyme and DNA carrier blanks were not run for every type of sample, and it should be stressed that such controls are essential in order to quantitate the results. These experiments require the isolation of relatively large amounts of AMV RNA. For example, in the one experiment described in detail we used 550 ml of AMV. One of the limiting factors still remains the supply of AMV despite the very generous supplies received from Dr. and Mrs. Beard via the Virus Cancer Program. We continue to be grateful for this large supplement to our own modest collection.

We have isolated poly(A) segments ranging from 30 to 120 nucleotides in length in other subsequent experiments, although the lower value was from our best experiment. It is possible that the length of poly(A) from different preparations of the same virus may differ, due to the state of the virus at the time of assembly in the cell. Other factors may also play a role in the size of the fragment isolated. For example, it has recently come to our attention that there may be a selective loss of poly(A)-RNA during extraction, unless chloroform is present in the phenol (32). Also the availability of the 70S or 35S RNA to the action of the two enzymes RNase A and T₁ may differ from

preparation to preparation due to the secondary structure or complexes formed by hydrogen bonding in the 70S unit. Our smallest value for poly(A) of 30 residues of adenylic acid was obtained from a 35S RNA isolated from a DMSO gradient in which hydrogen bonding was probably a minimal value.

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We have recently been informed by Drs. H. Rho and M. Green (personal communication) that they also find a poly A segment, at the 3'-OH terminus of the murine sarcoma-leukemia virus 30-40S subunit (unpublished data).

We have described the periodate - ^3H -borohydride labeling technique in detail. This method enables us to label as nucleoside trialcohols either a total nucleoside hydrolysate of RNA or just the terminal 3'OH nucleoside of an intact RNA molecule. From end and total nucleoside ratios we are able to determine the size and composition of the molecule under study. This method, perfected by the Randeraths, is good for small RNA molecules such as tRNA but less satisfactory for determination of the end of a molecule containing about 6,000 nucleotides, such as 35S viral RNA. For example, we must use a greater excess of both periodate and ^3H -borohydride, and it is necessary to assay more absorbance units of material in order to get enough counts in a single terminal nucleoside. This causes a) a higher borohydride "background" on the cellulose plates and b) in the 3'OH terminus assay, the plates are almost overloaded with respect to unhydrolyzed RNA. We are currently setting up a different separation method for the four trialcohols with the hope of achieving a lower background. This is the cation exclusion method of Singhal and Cohen (33) employing anion exchangers. We are using small aminex columns run at 50°, under pressure, with 50 μl samples.

Preliminary experiments indicate that we can achieve nice separations of the nucleoside trialcohols but we have not yet determined whether the problem due to high blanks has been solved.

(3) Isolation of poly(A) containing fragments from AMV RNA

Since we have found that all the poly(A) is at the 3'-OH terminus of "35S" RNA we can make use of methods aimed at the isolation of poly(A) (19,34). Selective digestion of the 35S RNA by specific enzymes, followed by isolation of a poly(A) containing fragment will enable us to isolate selectively nucleotides adjacent to the poly(A) region along with the poly(A). Current studies involve the use of ribonuclease A and ribonuclease T_1 for such

a purpose. For example, ribonuclease A in high salt concentrations hydrolyzes at U and C residues and should yield runs of A-3'OH with possibly some G and A nucleotides internally but no C or U. On the other hand, ribonuclease T₁ (which splits at G residues) should yield the terminal poly(A) plus internal U, C and A but no G. We have isolated such poly(A) containing fragments and presently are determining their size and composition by the periodate oxidation-H³-borohydride reduction technique both on terminal and total nucleotides.

There is as yet nothing definitive to report about nucleotides adjacent to this poly(A) segment except some preliminary evidence for an unusual number of guanylic acid residues. These were found following digestion by ribonuclease A.

It may be noted that on polio viral RNA Yogo and Wimmer (35) found the 3'OH terminus to contain 39 residues of poly(A) and two guanylic acid residues just internal to the poly(A) segment.

The bulk of the experiments described above have been carried out by Drs. Stephenson and Scott and in the earlier ones, Dr. LeR. Wirthlin.

c. Search for minor base constituents in high molecular weight RNA

As we discussed earlier, the possible functions of the oncogenic viral RNA are numerous. Many plant viral RNAs (TYMV and TMV, for example) and bacterial viral RNAs (QB and R₁₇) have the ability to accept a terminal 3'-OH adenosine using the nucleotidyl transferase which puts the terminal-CCA moiety on tRNAs, and in the case of plant viruses, the RNA can accept specific amino acids using aminoacyl synthetases (36,37). The evidence points to the possibility that these large RNA molecules terminate in a specific tRNA molecule of unknown function. These viral RNAs do not terminate in a run of poly(A), however, as do many of the viral RNAs from eukaryotic cells. Nevertheless the interesting possibility of having a specific tRNA molecule just interior to the 3'-OH terminal poly(A) segment is intriguing. We therefore decided to look for the presence of minor bases in both the 70S RNA complex (which is known to contain 3-4 tRNA molecules containing minor bases), and the "35S" RNA molecules freed of the 4S RNA by centrifugation through DMSO-sucrose gradients. As expected the analysis of the 70S RNA indicated the presence of some minor bases commonly found in tRNA. To our surprise however the "35S" RNA pooled fraction also contained unmistakable spots where some tritiated minor trialcohols of the bases are known to occur by radioautography on the two dimensional thin layer chromatograms. The amount of minor bases is several orders of magnitude lower per μ g RNA than that found in our tRNA control. The former has a molecular weight of approximately 2×10^6 compared with that of tRNA of about 2×10^4 . These experiments are technically much more difficult than determination of minor bases in tRNA, since the borohydride labeling method we use (20) is designed for only 1/100th as much material, and it is necessary to overload the plates with the four major base trialcohols in order to detect minor base trialcohols in the high molecular weight 35S RNA. The somewhat surprising possibility of traces of minor bases in "35S" RNA processed through DMSO gradients appears potentially important, and our preliminary evidence for their presence must be thoroughly substantiated. We are therefore continuing these experiments, and are accumulating more material for proper identification of the specific minor bases.

C. Other studies on post isolation labeling of oligonucleotides aimed at sequencing viral RNA

1. Introduction

Post isolation labeling techniques enable one to obtain terminal nucleosides which can be quantitated more readily because the amount of label introduced is of a higher order of magnitude than that from in vitro labeling of the viral RNA. Such techniques allow us to use smaller amounts of viral RNA for sequencing studies. This is particularly true of the high molecular weight RNA of the virus.

2. New methods for labeling the 3'-OH end and 5'P end of high molecular RNA with ^{32}P by chemical means

a. Attachment of ^{32}P to 3'-OH end

Preliminary experiments with new reagents which may be useful in sequential analysis of ribonucleic acid, in a manner similar to the Edman degradation of proteins, have been carried out. We have shown that attachment of a reagent to the 3'-OH-end of a polynucleotide by the formation of a 2', 3'-acetyl bond under anhydrous conditions can proceed in quantitative yields. Quantitative formation of ketals and acetals of nucleosides have been known (38). We have prepared and characterized the unlabeled and ^{32}P -labeled 5-hydroxypentanal monophosphate using trichloroacetonitrile as the condensing reagent (39).

5-Hydroxypentanal monophosphate reacted with mixed tRNA, nucleosides or nucleotides in dry formamide in the presence of triethylorthoformate to yield 2'-3'-labeled acetal as follows:

In the case of tRNA, formamide and excess reagents are dialyzed away or removed on a Sephadex G-100 column. The acetal bond is stable under basic pH's but is completely removed at pH 4.5, room temperature after ten hours. With E. coli B mixed tRNA, aminoacylation of lysine and isoleucine was quantitatively blocked after attachment of the reagent. Treatment at pH 4.5 for ten hours

restored the amino acid acceptance of lysine and isoleucine to 95 percent of that of the original mixed tRNA. The use of ^{32}P -labeled 5-hydroxypentenol monophosphate, which can easily be prepared in high specific activity of 10 mc/mmol (or more) is valuable in the identification and isolation of oligonucleotides from T_1 -ribonuclease digests corresponding to the 3' end pieces of the tRNA. It could also be useful in the 3'-labeling of high molecular weight RNA, which has already been shown to dissolve in formamide (40). The ^{32}P -phosphate monoester was easily removed by treatment of the labeled tRNA with alkaline phosphatase, without removing the reagent blocking the terminal 2', 3'-positions of tRNA.

b. Attachment of ^{32}P to 5' end

We also investigated possible reactions at the 5'-end of ribonucleic acid and utilized a phosphorimidazolidate intermediate for high specific activity labeling of the 5'-end of mixed tRNA. It is essential for this labeling procedure that the 5'-end of the molecule end in a phosphate, since phosphorimidazole will not label a free 5' hydroxyl group. Phosphorimidazolidate has been used as an intermediate in the synthesis of nucleoside-5'-triphosphates from the corresponding diphosphates (41,42). The internucleotide diphosphate ester is a weak nucleophile and was shown not to react with phosphorimidazolate or orthophosphate. The reaction of ^{32}P -labeled phosphorimidazolate or orthophosphate with tRNA (mixed) in anhydrous formamide in the presence of tributylamine led to the 5'-labeling of the tRNA. Excess reagents were dialyzed out and the tRNA retained lysine acceptance activity to at least 90 percent of its original acceptance activity. This

was a double labeling experiment using high specific activity ^3H -lysine and ^{32}P -phosphate.

The 5'- ^{32}P -label is useful in locating 5'-end fragments and can be removed by incubation with alkaline phosphatase after T_1 -ribonuclease digestion and isolation of oligonucleotides by chromatography on DEAE cellulose, in the presence of 7M urea and sodium chloride gradient elution. We are now trying to utilize this reaction for the 5'-end labeling of high molecular weight RNA followed by isolation and sequence analysis of the oligonucleotide which retains the label.

The two reagents described above have, as described, the ability to label with ^{32}P 3'-OH and 5'-P ends of high molecular weight RNA, and any internal oligonucleotides which one may produce and wish to label during the course of sorting out oligonucleotide fragments. As we have described in previous publications, covalent breaks in the 70S RNA terminate in 2'3' phosphate (1,2). By treating the 70S RNA with dicyclohexylcarbodiimide (Khorana, H.G., personal communication), we can convert such 3'-P termini to 2',3'-cyclic phosphate termini. After doing so, one could then label the only remaining phosphomonoester, namely, the 5'-P terminus (if it exists as such), with the ^{32}P -phosphorimidazole, and thus identify the 5'-P end of a high molecular weight molecule. It is also possible of course to label the 5'-end with a phosphokinase, as has been accomplished for Rous Sarcoma Virus by Silber *et al.* (43). In the case of the latter, the 5' terminus was found

to end in a hydroxyl group rather than as a 5'-P residue. Many high molecular weight RNAs however end in 5'-P and there is as yet no information on this point for AMV RNA.

D. Studies on the 4S RNA of AMV

1. Introduction

In addition to the investigations on the high molecular weight RNA of AMV the laboratory has been characterizing the 4S RNA found both free in the virion and that bound in the 70S RNA complex, the 70S associated 4S RNA.

Since little was known about the chemistry of the tRNA, a study was started three years ago to look at the minor base changes in the tRNA. Results have indicated that this tRNA is not simply an inclusion of normal bulk myeloblast tRNA within the envelope of the virus as it pinches off from the cell, but represents either a modification of the primary structure of host tRNA by this virus or synthesis of new tRNA by the virus, or specific selection of host cell RNA by the virions.

a. Minor bases in free 4S RNA of AMV

Three years ago we determined (44) the base composition of the free 4S RNA of AMV and compared it with normal chick liver and with infected myeloblast tRNA. The ^3H borohydride-labeling technique of the Randeraths previously described (15) was used for the identification of the major and minor bases. The findings reported here two years ago indicated that there were extensive differences in the various types of tRNA.

b. Minor bases in 70S associated 4S RNA of AMV

These experiments were extended to look at the 4S RNA which is complexed with the 70S RNA. This work has recently been published (45) and a reprint appended; therefore many of the details won't be included in this report. It was found that the minor base patterns of the 4S RNA were strikingly similar to that of the corresponding "free" 4S RNA of the virion. The percentage of minor bases of this 70S associated 4S RNA was lower than in free 4S RNA of the virion. It left open the possibility that not all of this 4S associated RNA might be transfer RNA (45). In an effort to establish whether the 70S associated 4S RNA satisfied the criteria of transfer RNA the amino acid acceptance patterns of these fractions have also been measured.

c. Amino acid acceptance of 70S associated 4S RNA of AMV

Last year we also completed a publication included herein which indicates that this RNA does have amino acid acceptor activity (46). The results from both the minor base content and amino acid acceptance indicated that the fraction contained several tRNAs and that possibly not all of the fraction was tRNA, although a great part of it appeared to be a mixture of transfer RNA molecules.

It is not known whether the tRNA binds to the 70S RNA during packaging of the virus particles as they bud from the host cells or whether the association occurs during rupture of the virion and isolation of the total RNA. As we

discuss in the proposal the function in the virus of tRNA in the 70S RNA complex and also the free tRNA was not known; however recent results of Dahlburg and Bishop (unpublished experiments) indicate that a specific tRNA (tRNA_{trypt}) present in the 70S associated 4S RNA acts as the primer for the reverse transcriptase. This finding of a specific tRNA in the reaction which enables the genetic material of the virus to be copied is exciting and indicates that tRNA might be involved in the regulation of virus production in addition to its established role in the regulation of the translation of proteins.

B. Normal reactions in the initial stages of protein synthesis. The recognition reaction between tRNA and its cognate aminoacyl tRNA ligase

1. Studies on lysyl tRNA synthetase - Dr. Hirshfield

The work in Dr. Hirshfield's laboratory is proceeding on two fronts, both of which are involved with the study of the aminoacyl-tRNA synthetases. Presently both of these studies are centered on lysyl-tRNA synthetase. One project involves the regulation of the synthesis of this enzyme, while the other is concerned with the mechanism by which lysyl-tRNA synthetase is able to interact selectively with its cognate tRNA. Both of these studies have evolved from a search for mutants of lysyl-tRNA synthetase from Escherichia coli which have a defect in their interaction with tRNA.

Studies on the regulation of lysyl-tRNA synthetase synthesis - Mutants of lysyl tRNA synthetase have been isolated from E. coli which appear to be regulatory (47,48). The activity of lysyl-tRNA synthetase in these mutants has been found to depend on the composition of the growth medium (47,48). In a minimal medium in which the mutant strains were isolated, the activity of lysyl-tRNA synthetase in various mutants is only 2% to 5% of that of the wild-type (47,48). Growth of the mutants in highly enriched broth results in an increase in lysyl-tRNA synthetase activity to wild-type levels (47). Further inquiry has shown that the enriched medium can be replaced by metabolites, the most potent being leucine peptides (49,50). These compounds when added alone to minimal medium at 3 mM concentration can stimulate lysyl-tRNA synthetase activity to wild-type levels in all mutants tested (48,50). The peptides also enhance lysyl-tRNA synthetase activity 2- to 3-fold in wild-type E. coli (50). Importantly, experiments indicate that all conditions (rich media, metabolites) which stimulate the activity of lysyl-tRNA synthetase in the mutants do so by enhancing the synthesis of the enzyme rather than by activating inactive precursors (49,50).

These results are significant for two reasons. First, they suggest that the synthesis of lysyl-tRNA synthetase, which is a constitutive enzyme, can be under the control of a metabolite. According to current thinking, metabolites are thought to exert little influence on the synthesis of constitutive enzymes (51). Second, these results suggest that leucine peptides, particularly dipeptides, may play a role in enzyme regulation, in at least the bacterium E. coli.

Studies on mutant lysyl-tRNA synthetase - A number of strains of E. coli have been isolated which have a low level of lysyl-tRNA synthetase activity. These mutants were all isolated as slowly growing strains resistant to an analog of lysine-thiosine which inhibits growth of wild-type E. coli. The lysyl-tRNA synthetases from these mutant strains all have an altered structure, for they are all more thermally stable than the wild-type enzyme. None of these mutant enzymes show a large change in their kinetic parameters such as the K_m for lysine, ATP and tRNA. Thus by selecting for mutants by thiosine resistance it has not been possible to isolate mutants of lysyl-tRNA synthetase which are

defective in their interaction with tRNA.

We have decided to examine more closely the mutant enzymes that have been isolated. The finding that the mutant enzymes are more thermally stable than the wild-type enzyme is a rare event, for most mutant enzymes are more thermolabile than their wild-type counterpart. Therefore we have begun to isolate and purify the wild-type and one mutant enzyme, to study them more intensively. One of the major initial goals is to fingerprint the mutant and wild-type protein to detect what amino acid change(s) has occurred. Working out the fingerprinting techniques now will facilitate analysis of mutant enzymes found in the future. A second point of interest is to learn whether the increase in thermal stability in the mutant enzyme is due to a stronger interaction between the subunits of the enzyme.

In our initial studies we have used the method of Waldenström (52) to purify lysyl-tRNA synthetase. We have obtained the same results as Waldenström with wild-type enzyme down to the penultimate step in the purification. However, purifying the enzyme further results in its partial or complete inactivation, even though by the criterion of polyacrylamide gel electrophoresis the enzyme is highly purified. Hence it is necessary to determine what is causing the inactivation. We have also begun the process of comparing mutant and wild-type enzymes and find that on DEAE columns the mutant enzyme elutes later (~10% of the gradient volume) than the wild-type enzyme.

2. Chemical studies on the molecular nature of the recognition reaction between aminoacyl-tRNA synthetase and its cognate tRNA - D. Coleman

We have been seeking to determine the specific role of sulfhydryl groups in methionyl-tRNA synthetase (MRS) by studying the mode of inactivation of the enzyme by sulfhydryl reagents. Particularly, we wished to determine whether loss of the ability to incorporate methionine into tRNA which results from treatment with p-chloromercuribenzoate (PCMB) was related to an inability to form an enzyme-tRNA complex or to an inability to carry out the transfer from methionyl-adenylate. On incubation in 10 mM PCMB, total inactivation occurred instantaneously. Since the enzyme could not activate methionine, we first wished to determine whether the enzyme could transfer methionine to tRNA if it were provided with pre-formed methionyladenylate. Labelled methionyl-AMP was prepared following Eldred and Schimmel (53) by incubating [^{14}C]-methionine and ATP with the enzyme and separating the methionyladenylate:enzyme complex from the excess substrates. On addition of tRNA, methionine was incorporated, demonstrating that the methionine remained activated during the procedure. Met-AMP could be dissociated from the enzyme by acid denaturation followed by neutralization. When such a preparation of met-AMP was incubated with PCMB-inactivated enzyme and tRNA we observed no formation of methionyl-tRNA. Thus, part of the effect of PCMB directly affects the ability of the enzyme to transfer methionine to tRNA irrespective of whether the methionine activation capability has been affected. To analyze further these results, the binding of various substrates to PCMB-MRS was examined. tRNA binding was studied using the nitrocellulose filtration technique of Yarus and Berg (54). The results indicated that the extent of PCMB-enzyme-tRNA complex formation was less than 50% of that observed with the native enzyme. A study of enzyme-tRNA complex formation by molecular exclusion chromatography using buffers containing tRNA (55) gave no indication of binding of tRNA to PCMB-MRS. We suspect that the residual "complex formation" observed with the nitrocellulose filter method represents non-specific binding of tRNA to inhibited enzyme. The use of molecular exclusion chromatography represents a true equilibrium situation. Further,

since unfractionated *E. coli* tRNA is used, binding of tRNA^{met} represents selective binding, with all the other tRNAs competing with tRNA^{met} for any non-specific binding sites of the enzyme.

When the binding of methionine to MRS was examined by means of equilibrium dialysis, we again found that, at saturation, approximately half as much methionine bound to the inhibited enzyme as to the control enzyme. Hence, MRS which has been completely inhibited by PCMB cannot bind tRNA and has a diminished affinity for methionine.

It is of particular interest to investigate whether the PCMB blocks the tRNA binding sites directly or exerts its effect via a conformational change of the enzyme. We have investigated the effect of PCMB on the intrinsic fluorescence of MRS and found that nearly 80% of the fluorescence of the native enzyme is quenched over a ten-minute period. However, as indicated in Table VII, the initial loss of both exchange and incorporation activities is much more rapid than the loss of the fluorescence. Thus, the initial loss of activity does not appear to be directly related to a far-reaching conformational transition. It seems likely that the initial inhibition may arise from the reaction of one or more critical sulfhydryl groups with PCMB. Plans for pursuing this question further are described in the proposal.

We have also carried out a study of the kinetics of inactivation of MRS by a reagent which reacts with tryptophan residues, dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide (DSB) (56). Although DSB can also react with sulfhydryl groups, the inactivation kinetics differ significantly from those seen with PCMB in that DSB causes inactivation of incorporation activity at a greater rate than inactivation of exchange activity (see Table VIII). We did not observe this effect using PCMB. The presence of methionine and ATP results in a dramatically lowered rate of inactivation of the exchange activity while its effect on the incorporation activity is considerably smaller (Table IX). On the other hand, the presence of tRNA decreases the rate of inactivation of the incorporation activity but has little effect on the exchange activity (Table IX). It is worthy of note that the tRNA protective effect does not decrease the rate of loss of incorporation activity to less than that of the exchange activity, a finding which is consistent with a model for the enzyme activity in which exchange capability is necessary, but not sufficient condition for incorporation activity. The use of reagents such as DSB, PCMB, and others which have yet to be screened provide useful probes with which we can dissect the parameters affecting the activity of this representative of a vital class of enzymes.

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IV. Publications from the Huntington Laboratories pertaining to work supported in part by the contract. The publications cover two years - 1972-1973 and 1973-1974, since last year we were not required to submit a formal proposal due to changes in starting dates of contract.

1971-1972

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Table I

Comparison of nucleoside trialcohols released from terminally labeled viral RNA following hydrolysis by piperidine and Ribonuclease A

"35s" RNA

Piperidine hydrolysis - yields total terminal nucleoside

	CPM/ μ g RNA	Per cent of terminal nucleoside
A	412.6	88.4
C	15.7	3.4
G	12.9	2.7
U	25.6	5.5
	<u>466.8</u> - Total terminal nucleoside	<u>100.0</u>

Pancreatic Ribonuclease A hydrolysis

	CPM/ μ g RNA	Per cent of total terminal nucleoside (466) released by piperidine hydrolysis
A	34.0	7.3
C	11.8	2.5
G	4.6	1.0
U	5.7	1.2
	<u>56.0</u>	<u>12.0</u>

7% of ends terminate in Py-A, 93% could be G-A or A-A.

b.

Table II

Comparison of nucleoside trialcohols released from terminally
labeled 70s RNA following hydrolysis by piperidine and
Ribonuclease T1

70s RNA

Piperidine Hydrolysis

	$\mu\text{moles}/\mu\text{g RNA}$	Per Cent
A	.185	53.6
C	.083	24.0
G	.041	12.2
U	.035	10.2
	<hr/>	
	.344	

T1 Ribonuclease Hydrolysis

Per cent of total control
terminal nucleoside released
by piperidine (.344)

A	.0092	2.7
C	-	-
G	.0052	1.5
U	.0045	1.3
	<hr/>	
	.0189	5.5

Very little A was released; therefore G-A could not be the penultimate nucleotide.

Table III

Comparison of total and 3'-OH-terminal nucleosides from AMV "35s" RNA and poly(A) fractions

Untreated					Phosphatase Treated				
		Total		3'-OH End		Total		3'-OH End	
		CPM	Per Cent	CPM	Per Cent	CPM	Per Cent	CPM	Per Cent
"35s" RNA		③		②		⑤		④	
	A	53,500,000	26.9	33,600	84.3	56,800,000	28.5	58,200	64.8
	C	52,700,000	26.5	1,150	2.9	44,500,000	22.4	16,700	18.6
	G	52,100,000	26.2	2,540	6.4	50,700,000	25.5	6,390	7.1
	U	40,500,000	20.4	2,560	6.4	46,900,000	23.6	8,510	9.5
Total		199,000,000		39,900		199,000,000		89,800	
PolyA from "35s" RNA		⑧		⑦		⑩		⑨	
	A	1,310,000	100.0	44,900	98.8	1,290,000	98.5	42,500	89.9
	C	0*	0	0*	0	0*	0	1,580	3.4
	G	0*	0	537*	1.2	20,300	1.5	1,180	2.5
	U	0*	0	0*	0	0*	0	1,190	4.2
Total		1,310,000		45,400		1,310,000		46,500	

Legend to Table III: Taken from (2)

The counts indicated were obtained from individual two dimensional cellulose plates separating the four nucleoside trialcohols indicated by added carrier "cold" trialcohols visualized in UV light. Because of high blanks and spurious "hot" spots, great care was taken to cut out all the surrounding areas in addition to the known trialcohols. Approx. 120 samples were cut from each plate in order to determine the cpm in the trialcohols. The samples marked with * were too close to the average surrounding background samples to warrant any significance. Samples not so marked have an overall counting accuracy of $\leq \pm 5\%$.

All counts have been corrected for the DNA blanks which were worked up in an identical manner using individual plates. These were small corrections but essential for estimating in the polyA samples the presence or absence of nucleosides other than adenosine. Aliquot sizes have been taken into account and also all cpm normalized to the total base #1 and #6 indicated in Fig. 1 which were from samples never precipitated from alcohol following borohydride treatment. All other samples were precipitated from alcohol 5 times in order to wash the RNA free of [^3H] borohydride used for the 3'-OH determinations (24). The correction of these samples varied as follows: 2 and 3, 1.40; 4 and 5, 0.87; 7 and 8, 11.2; and 9 and 10, 2.55. These were due to losses incurred during the reprecipitation.

Table IV

Amount of poly(A) present in "35s" RNA from AMV
(Data taken from Table III)

Per cent of "35s" in poly(A)

$$\frac{\text{Total nucleoside in poly(A)} \textcircled{8}}{\text{Total nucleoside in 35s} \textcircled{3}} = \frac{1,300,000}{199,000,000} = 0.65$$

Per cent of the adenosine of "35s" in poly(A)

$$\frac{\text{Total A in poly(A)} \textcircled{8}}{\text{Total A in "35s"} \textcircled{3}} = \frac{1,310,000}{53,500,000} = 2.5 \text{ per cent}$$

Comparison of terminal nucleosides

$$\frac{\text{Total terminal nucleoside in poly(A)} \textcircled{7}}{\text{Total terminal nucleoside in "35s"} \textcircled{2}} = \frac{45,400}{39,900} = 1.1$$

$$\frac{\text{Total terminal A in poly(A)} \textcircled{7}}{\text{Total terminal A in "35s" RNA} \textcircled{2}} = \frac{44,900}{33,600} = 1.3$$

Table V

Approximate size of the "35s" RNA and the poly(A) isolated from it
(Data taken from Table III)

Original "35s"

I A. Control

$$\frac{\text{Total nucleotides } ③}{\text{Total ends } ②} = \frac{199,000,000}{39,000} = 4,992 \text{ nucleotides} \times 350 \text{ (ave. wt. per nucleotide)} = 1.75 \times 10^6 \text{ ave. mol. wt.}$$

B. Phosphatase treated control

$$\frac{\text{Total nucleotides } ⑤}{\text{Total ends } ④} = \frac{199,000,000}{89,800} = 2,215 \text{ nucleotides} \times 350 = 775,000 \text{ ave. mol. wt.}$$

C. Since phosphatase ends contain the control ends also:

$$\frac{\text{Phosphatase ends } ④ - \text{Control ends } ②}{\text{Control ends } ②} = \frac{89,800 - 39,900}{39,900} = 1.25$$

$$\frac{\text{Phosphatase terminal A } ④ - \text{Control terminal A } ②}{\text{Control terminal A } ②} = \frac{58,200 - 33,600}{33,600} = 0.74$$

Poly(A) Isolated from "35s"

II A. Control

$$\frac{\text{Total nucleotides } ⑧}{\text{Total ends } ⑦} = \frac{1,310,000}{45,400} = 28.9 \text{ nucleotides} \times 350 = 10,100 \text{ ave. mol. wt.}$$

$$\frac{\text{Total adenosine } ⑧}{\text{Total terminal adenosine } ⑦} = \frac{1,310,000}{44,900} = 29.2 \text{ nucleotides} \times 350 = 10,200 \text{ ave. mol. wt.}$$

B. Phosphatase treated poly(A)

$$\frac{\text{Total nucleoside } ⑩}{\text{Total ends } ⑨} = \frac{1,310,000}{46,500} = 28.1 \text{ nucleotides} \times 350 = 9,860 \text{ ave. mol. wt.}$$

$$\frac{\text{Total adenosine } ⑩}{\text{Total terminal adenosine } ⑨} = \frac{1,290,000}{42,500} = 30.3 \text{ nucleotides} \times 350 = 10,600 \text{ ave. mol. wt.}$$

C. Since phosphatase ends contain the control ends also:

$$\frac{\text{Phosphatase ends } ⑨ - \text{Control ends } ⑦}{\text{Control ends } ⑦} = \frac{46,500 - 45,400}{45,400} = .02$$

$$\frac{\text{Phosphatase terminal A } ⑨ - \text{Control terminal A } ⑦}{\text{Control terminal A } ⑦} = \frac{42,500 - 44,900}{44,900} = -.05$$

Table VI

Approximate Size of "35S" RNA and Poly(A) Isolated from it

Data Taken from Table III

"35S" RNA	Control	Phosphatase treated
Nucleotides	5,000	2,220
Av. mol. wt.*	1.75×10^6	0.77×10^6
Poly(A)	Control	Phosphatase treated
Nucleotides	28.9	28.1
Av. mol. wt.	10,100	9,860
Adenosine	29.2	30.3
Av. mol. wt.	10,200	10,600

*X 350 the average molecular weight per nucleotide.

Table VII. Effect of PCMB* on activity and fluorescence of methionyl
tRNA synthetase in 0.01 M TES** buffer pH 7.5

Reaction time (min)	Incorporation activity (%)	Exchange activity (%)	Fluorescence (%)
0.25	40	37	-
0.5	-	-	77
2.0	30	26	52
5.0	21	22	31
10.0	14	21	24
20.0	9	17	-

*PCMB = p-chloromercuribenzoate

**TES = (N-tris [hydroxymethyl]methyl-2-aminoethane sulfonic acid)

Table VIII. Effect of DSB* on activity of methionyl-tRNA synthetase in
0.1 M phosphate, pH 7.5

Reaction time (min)	Incorporation activity (% of control)	Exchange activity (% of control)
0.25	22.2	44.8
2.0	4.7	16.5
4.0	3.3	12.8
6.0	2.6	11.7
8.0	1.9	11.2

Table IX. Effect of substrates on inactivation of methionyl-tRNA synthetase by DSB*

Buffer	Substrate	Concn.	Incorporation activity (% of control)	Exchange activity (% of control)	<u>Incorp. activity</u> <u>Exchange activity</u>
1.	Met + ATP	0 mM	4	16.5	0.24
		2 mM	12.5	39.3	0.32
		4 mM	13.7	50.5	0.27
2.	tRNA	0 µg/µl	9.8	26.0	0.37
		2 µg/µl	25.0	29.7	0.84
		4 µg/µl	19.0	26.0	0.71

Activities measured after reaction with DSB for 2.0 min

Buffer 1: 0.1 M phosphate, pH 7.5

Buffer 2: 0.1 M imidazole, pH 6.0

For Tables VIII and IX: *DSB = dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium
bromide

Figure 1

Comparison of nucleoside trialcohols released from terminally labeled RNA following hydrolysis by different methods

	Mode of Action		If Viral RNA terminating in labeled A	
	N _x	A*	N _x	A*
Control - piperidine -	↓P	↓	↓P	↓
	all mononucleotides		all the rest mononucleotide	labeled A trialcohol

Ribonuclease A

Splits at pyrimidine 3'P

example

If RNA ended in C-A or U-A
labeled trialcohol A released

example

If RNA ended in A-A or G-A
no labeled trialcohol released

Ribonuclease T1

Splits at any G

If RNA ends in G-A
labeled trialcohol A released

If RNA ends in anything but G-A
no trialcohol A released

On the chromatography used mononucleotides and oligonucleotides stay at the origin, only the nucleosides or nucleoside trialcohols move from origin.

Figure 2

Schematic diagram of experimental plan to determine amount of poly(A) in 35s RNA from AMV