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Title: THE BIOLOGICAL SIGNIFICANCE OF LOW MOLECULAR WEIGHT RNA ASSOCIATED WITH TUMOR VIRUS

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RNA isolated from BAI strain A (Myeloblastosis) Avian Tumor Virus can be fractionated into two fractions on a sucrose density gradient, one of higher molecular weight and one of low molecular weight. The low molecular weight (LMW) virus RNA was found to chromatograph the same as E. coli tRNA on a DEAE cellulose column and to have elution properties similar to myeloblast tRNA on a MAK column. A portion of the LMW virus RNA fraction was demonstrated to have the ability to accept amino acids in an energy requiring, aminoacyl-synthetase catalyzed reaction. The acceptance exhibited toward nine amino acids tested individually varied considerably. The pattern of amino acid acceptance shown with LMW virus RNA did not correspond to that seen with myeloblast tRNA. Finally, the LMW virus RNA fraction was shown to transfer accepted amino acids to a growing peptide chain in a cell-free polysome system. The LMW virus RNA fraction was, thus, characterized as transfer

RNA. The presence of a transfer RNA possessing a modified pattern of amino acid acceptance is of significance in terms of translational control of protein synthesis.

The Biological Significance of Low Molecular Weight RNA Associated with Tumor Virus

by

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INTRODUCTION

Cellular Transfer RNA

Since its discovery in 1957 (Hoagland, Zamecnik, and Stephenson, 1957), amino acid transfer RNA (tRNA)[1] or soluble RNA (sRNA) has been shown to play a central role in the translational mechanism of protein synthesis. It has also been suggested that tRNA may be involved in the regulation and coordination of the synthesis of groups of related proteins (Ames and Hartman, 1963).

The sequence of amino acids in a protein is determined by a sequence of nucleotides in a messenger RNA (mRNA) which in turn has been transcribed from a sequence of nucleotide pairs in a segment of DNA. The sequence of the mRNA is translated in a stepwise process. The amino acids are specified by at least one triplet of nucleotides (codons) in the mRNA and are added to the growing

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[1] List of abbreviations used throughout this paper: tRNA, transfer ribonucleic acid; sRNA, soluble ribonucleic acid; mRNA, messenger ribonucleic acid; DNA, deoxyribonucleic acid; KAc, potassium acetate; EtOH, ethanol; NaCl, sodium chloride; DEAE, diethyl aminoethyl; EDTA, ethylenediaminetetraacetic acid; KCl, potassium chloride; ATP, adenosine-5'-triphosphate; $MgCl_2$, magnesium chloride; TCA, trichloroacetic acid; NH_4OH, ammonium hydroxide; NaOH, sodium hydroxide, HCl, hydrochloric acid.

polypeptide chain one at a time. The attachment of the specific amino acid to the growing polypeptide chain takes place in three stages on the ribosome. In the first, each amino acid is attached to a specific tRNA molecule by a specific enzyme, an aminoacyl-tRNA synthetase. In the next stage, the aminoacyl-tRNA specified by the next vacant codon binds to the mRNA-ribosome complex. In the third stage, the aminoacyl-tRNA complex is transferred to the carboxyl-terminal end of the growing polypeptide chain, and the tRNA's released from the ribosome (Nakamoto et al., 1963; Brown and Lee, 1965; Watson, 1965). In this process the tRNA has been used as an adaptor molecule which recognizes both the message on the mRNA (the codon) and a specific amino acid to match that codon.

Structurally the tRNA molecule must have at least two sites of recognition. The recognition of the codon base triplet is thought to be due to hydrogen bonding to a corresponding nucleotide triplet (or anti-codon) on the tRNA molecule. The recognition of the specific amino acid is not well understood. It is possibly due to the specific tertiary structure of the tRNA and the aminoacyl-tRNA synthetase (Fresco et al., 1966).

Transfer RNA, like all ribonucleic acid, is made up of a phosphate-ribose chain with four different side groups; the purines--adenine and guanine, and the pyrimidines--uracil and cytosine. Each of the tRNA's are made up of approximately 80 nucleotides in a single

chain with a molecular weight of about 25, 000. In addition to the usual nucleosides, tRNA has been shown to contain large proportions of unusual nucleosides including inosine, thymine riboside, dihydrouridine, pseudouridine, thionucleosides, and methylated bases (Gold and Hurwitz, 1963; Brown and Lee, 1965; Lipsett, 1966).

Although the exact base sequence of all the various tRNA's has not been determined, several species including alanine, tyrosine, serine, and phenylalanine tRNA's have been studied extensively (Cantoni et al., 1963; Zachau et al., 1966). The bases are so arranged that a certain percent of the molecule can be hydrogen bonded between matching bases to give a double helical structure with the chain folding back on itself (Doty et al., 1959). Several possible configurations exist all of which include some loops in the chain made possible by the unpaired bases and unusual nucleosides. Disulfide bonds have also been shown to play a role in the establishment of secondary structure (Lipsett, 1966). Several of the unusual nucleosides have stronger binding characteristics than the normal hydrogen bonding of the usual nucleosides. One of the loops is felt to be the site of the anti-codon sequence. The 5'-phosphate-terminal base was found to be guanine 80% of the time (Bell, Tomlinson and Tener, 1964), and the 3'-hydroxyl-terminal ends in a pCytosine-pCytosine-pAdenine sequence (Hecht et al., 1958; Daniel and

Littauer, 1965; Lebowitz et al., 1966). The terminal adenine is the site of attachment of the specific amino acid where it forms an ester linkage to the 2'- or 3'-hydroxyl group of the base (Zachau, Acs and Lipmann, 1958).

The exact tertiary structure of tRNA is not known (Fresco et al., 1966). Hydrogen bonding experiments have shown that 82% of the nucleotides are involved in a helical structure (Englander and Englander, 1965) and it has been suggested that the nucleotides are involved in three structural forms: those in the formation of a "DNA-like perfect" double helix, those not hydrogen bonding, and those involved in an "imperfect" helix (Kisselev et al., 1964). Whatever the exact tertiary structure may be, it must be complex enough to carry out the recognitions required with slight differences in conformation for each tRNA species.

tRNA Control Systems

In 1961, F. Jacob and J. Monod (1961) presented a model for the regulation of protein synthesis that has served as the basis for study of the subject since that time. They proposed that each gene, or DNA cistron, acts as a template for the synthesis of mRNA from which the polynucleotide corresponding to that cistron is transcribed. A group or set of cistrons which are all closely linked on the genetic map form an operon and are all coordinately controlled by a common

gene called an operator. The operator can be "closed," impaired by a repressor substance, in which case the operon will transcribe no mRNA for any of its cistrons. Or the operator can be "open" when no repressor is impairing its function and mRNA is transcribed by the operon for all its cistrons. For a very short time after formation the mRNA serves as the template for the cistronic polynucleotide sequence specified by the DNA operon. It is then broken down by cellular nucleases and the nucleotides returned to the cell nucleotide pool (Stent, 1964).

Not all of the proteins coded for on a given mRNA are necessarily produced at the same frequency or to the same extent. This phenomenon is called polarity (Martin et al., 1966), and the Modulation Model (Itano, 1957; Ames and Hartman, 1963) was proposed to explain it. In this model a modified tRNA can serve as a modulator and control peptide synthesis in the following ways: 1) by affecting the ribosome-aminoacyl-tRNA complex in such a way as to result in a high probability to dissociate, or 2) by limiting the rate of synthesis by binding tightly to the mRNA. Sueoka and Kano-Sueoka (1964; Roth et al., 1966; Sueoka, Kano-Sueoka and Cartland, 1966) state that modification of tRNA could be in several forms:

1. Anticodon Sites
    a. Loss or Activation of Codon Recognition
    b. Change of Codon Specificity

2. Enzyme Site

   a. Loss or Activation of Amino Acid Acceptor Activity

   b. Specificity Change of Amino Acid Acceptor Activity

3. Ribosome Site

   a. Failure or Recovery of Binding on Ribosome

There are many accounts of modified tRNA. One of the most frequently studied modifications is methylation of the bases. The transfer ability of phenylalanine- and leucine-tRNA has been shown to be altered by the presence or absence of methylated bases (Littauer, Revel and Stern, 1966; Peterkofsky, Jensensky and Capra, 1966; Wainfan, Srinivasan and Borek, 1966). Methylation can cause a change in secondary and tertiary structure sufficient to alter the specificity of recognition sites. Littauer et al. (1963) have shown that tRNA containing methylated bases have a greater sensitivity to exonucleases. This would cause a shift in the availability of the adaptor tRNA molecule to the system.

It has been shown that formylated methionyl-tRNA in bacterium is closely associated with protein synthesis initiation (Marcker, 1965; Dickerman et al., 1966; Marcker, Clark and Andreson, 1966). In a large percentage of protein synthesis studies, methionyl-tRNA was found to be the first attached to the mRNA. Formylation of this tRNA then occurred and synthesis continued on down the mRNA. There are at least two species of methionyl-tRNA and only one of

these is capable of being formylated. A modification of the formylating characteristics of this species could effect the efficiency of the synthesis initiation.

The triplet code is said to be degenerate, i.e., there is more than one codon for each amino acid. Many of the 20 amino acids have been shown to be transferred by more than one species of tRNA (Sueoka and Yamane, 1962; Bennett, Goldstein and Lipmann, 1963; Bennet, Goldstein and Lipmann, 1965; Weisblum et al., 1965; Bergquist, 1966; Wetterstein, 1966). A modulator, therefore, does not necessarily have to be a modified tRNA, but simply an already existing minor species.

There are 64 codons allowed by the triplet sequence message. At least 20 of these codons would have to be reserved for the 20 standard amino acids. Assuming each codon has a particular species of tRNA, this leaves a possible 44 codons to be used as modulators. As Stent (1964) points out, however, this could not be a one to one modulation (one codon to one operon) since many more than 44 operons are present in most organisms. Two possibilities are suggested to regulate a large number of operons. One of these is that the modulating codons would have more than three nucleotides involved in the mechanism. The second would involve two or more modulating codons for each operon. Both of these possibilities could regulate many more than 44 operons.

Since tRNA is directly responsible for amino acid incorporation into a peptide, the availability of the various tRNA species is essential for normal protein synthesis. The level of concentration of tRNA adaptor would control the rate of peptide formation in much the same manner as an enzymatic reaction. If the concentration for even a single amino acid was lowered significantly, the overall synthesis rate would also be lowered. Thus a modification of tRNA could effect synthesis by altering the effective concentration of active tRNA. Such a modification could conceivably increase or decrease the effective tRNA concentration. If normal, active tRNA is rendered inactive as an adaptor, synthesis would decrease. If an already inactive species, possibly one of the modulators or minor species of tRNA, is changed in such a way as to become active as an adaptor, synthesis would increase.

Viral Systems Having Altered tRNA's

Several investigators have shown that new tRNA species appear and that the relative concentrations of the tRNA's change in the cell upon virus infection. Leucyl-tRNA from phage infected *E. coli* B has been shown to be altered (Kano-Sueoka, 1966; Sueoka, Kano-Sueoka and Gartland, 1966). A time study of phage infection shows a new species of leucyl-tRNA being formed.

Cells infected with Herpes virus have a different concentration

of tRNA than uninfected cells (Subak-Sharpe et al., 1966). It is suggested that since the viral genome is different from the cell genome, it is reasonable to believe that the virus will need a different concentration of the various tRNA's in order to replicate its genome. The observed difference in tRNA concentration in the infected cell is the result of the compensation for the viral genome difference and is caused by the virus in some manner.

The hepatic carcinogen, ethionine, has also been shown to cause a change in the leucyl-tRNA pattern in rat liver tissue (Axel, Weinstein and Farber, 1967). Two of the normally three leucyl-tRNA species are deleted when ethylation occurs.

Sporulation, a radical morphogenesis, has been studied in regard to tRNA patterns in Bacillus subtillis (Doi and Kaneko, 1966). It was found that a third serine specific tRNA was present in the sporulating organism compared to two species in the non-sporulating individual. This suggests that altered tRNA species may play a role in radical cell changes such as sporulation and virus induced alterations.

In addition, it has been shown that tumor tissue contains altered tRNA methylase (Borek, 1963; Tsutsui, Srinivasan and Borek, 1966). Such altered tRNA methylase could cause alteration of tRNA in the infected cell.

Avian Leukosis Virus

The virus-induced tumors of the chicken make up a group of neoplastic diseases that have pathogenic aspects ranging from leukosis to simple fibrosarcomas. A list of these neoplasms would include fibrosarcoma, myxosarcoma, osteochondrosarcoma, endothetcoma, hemorrage disease, lymphomatosis, osteosarcoma, carcinoma, myelocytic tumors, and spindle cell sarcoma (Beard, 1963). Many other virus-induced, solid tissue tumors (sarcomas), have since been found. Early investigators considered the agents of these diseases to be entities specific for the tissue from which they were isolated. As work progressed, however, the idea of specificity has lessened and marked exceptions are being found to the rule. Although viruses that have been isolated from one type of neoplastic tissue have been found to induce a completely different type of neoplasm, several definite strains of avian tumor viruses have been immunologically determined and established. Although all of the possible interactions between agent and neoplasm have probably not been illucidated, some of the multiple responses have been established (Beard, 1963).

Strain R originated in a bird with pure erythroblastosis. The strain has been passed through many generations and is capable of inducing erythroblastosis and lymphomatosis in birds that do not

first die of the leukemia.

Strain ES4 was isolated from chickens with myeloblastosis and associated fibrosarcoma. This strain is now carried as two sublines, one derived from the transplantation of the sarcoma and the other from inoculation of blood from those with the leukemia. The major response of the later is erythroblastosis, with both sarcoma and the leukemia appearing as a response of the first subline. Renal carcinoma has also been observed to be associated with this strain. Strain 13 is quite similar to ES4.

RPL strain 12 is one of the more diverse forms. It was originally isolated from a tumor resembling lymphosarcoma in a bird that revealed no evidence of leukemia. It is now known, that although lymphomatosis is the primary response, erythroblastosis, osteopetrosis, sarcoma, and hemangiomatosis also result from this agent.

The Rous sarcoma, discovered in 1910, is primarily responsible for causing fibrosarcoma. It also seems possible that through proper manipulation the Rous virus can cause lymphomatosis and erythroblastosis, as well as certain endothelial diseases.

In 1941 the BAI strain A was derived from two birds with neurolymphomatosis, neither of which showed any leukemia. This strain now causes myeloblastosis, lymphomatosis, osterpetrosis, sarcoma, and renal carcinoma. This strain is unique in that it is

the only established strain that induced myeloblastosis and is considered to be a pure strain of myeloblastosis virus.

Several general statements can be made regarding the pathogenesis of these strains. A given disease does not differ in principle regardless of the inducing agent. Lymphomatosis and erythroblastosis seem to be the most common response to all strains. In contrast there are some definite differences in the established strains. If a single virus is defined as an entity of fixed and invariable properties, then it is obvious that all of the neoplasms are not due to a single agent. But since the test involves hosts that are themselves variable and not a single entity, it is impossible to say that the host response is an absolute criterion for the virus potential. Therefore, it can only be said that the chicken tumors comprise a group of diverse yet related growths that result from a family of different yet related viruses.

The virus used in this study is the BAI strain A tumor virus. The general morphology of the virus is fairly well understood and resembles closely the morphology of the Rous sarcoma virus (Beard et al., 1963; Bonor et al., 1963; Bonor, Heine and Beard, 1964). The shape is generally considered to be spherical with a diameter of 120-140 millimicrons, although it can take on rather odd shapes depending on the method of preparation. The virus is an RNA virus whose main nucleic acid constituent is of the ribose form. Very

little, if any, DNA is found. By staining, nucleic acid is found in the central nucleoid and is closely surrounded by an inner membrane which remains in association with the nucleoid when the outer membrane or coat is removed. The outer membrane is rather ill defined with some suggestion of peripherial knobs associated. Between the two membranes is the intermembrane material that is less dense than both membranes. It is probably a distortion of this material that results in the odd shape under some isolating procedures (Bernhard et al., 1958; Bonar and Beard, 1949; Bader, 1964).

It has been shown (Beard et al., 1963) that the virus has a low hydrated density of 1.059 and a rather high water content of 80% by volume. It is made up of about 60% protein, 35% lipid, 2% RNA (with an equivalent molecular weight of about 9.8×10^6) and small amounts of carbohydrates (Bonar, Heine and Beard, 1964). The viral RNA can be fractionated after isolation into two distinct high and low molecular weight components (Bonar et al., 1967; Robinson, Robinson and Duesberg, 1967).

The Primary Host Cell; Chick Myeloblasts

Although, as mentioned above, the BAI strain A virus infects several different tissues throughout the body of the host, the target of primary importance in this study is the myeloblast cell. Several theories concerning the developmental interrelationship of the blood

cell exist with the monophyletic and polyphyletic theories being the most popular (Wintrobe, 1961; Bloom and Fawcett, 1964). According to the monophyletic or "unitarian" theory of hemopoiesis, all of the blood elements of the adult originate from one common stem cell, the hemocytoblast, and according to the polyphyletic theory, the completely differentiated adult blood cells have individual precursors. Regardless of which theory one accepts, the "blast" cell of interest is the precursor to the myelocytes and thus, to the neutrophils, eosinophils, and basophils of the granulocytic series of leukocytes (Wintrobe, 1961).

The myeloblast cell is found normally only in the hemopoietic tissue and not in the peripherial blood. The cells are in a very active stage of growth and division occurs frequently. Since they are in a dynamic growth condition developing into myelocytes, the identifying characteristics of a given phase are arbitrary. Some investigators (Bloom and Fawcett, 1964) maintain that there is very little clinical difference among the "blast" cells for all three leukocytic series. However, for the purpose of classification, the myeloblast has been described carefully (Wintrobe, 1961). It possesses a relatively large nucleus and a small amount of cytoplasm. The chromatin is evenly diffused throughout the nucleus, but from 2-5 nucleoli can be seen. The cytoplasm is basophilic and there is no granulation. The cell is somewhat motile and ranges in size from 10 to 20

microns in diameter (Lucas and Jamroz, 1961).

Virus Infection and Growth

Although it has been well established that the virus is the causative agent in myeloblastosis, the actual mechanism for halting cell maturation is not known. Much work is presently being done on the biochemical interactions of the virus and its host cell. Studies stemming from both electromicroscopy and chemical experiments, have described some of the structural changes (Beard et al., 1963).

The virus enters the host cell and, in some manner, causes the production of virus progeny. The new viruses are released through the cell membrane in a "budding" fashion, taking along part of the cell membrane as the outer virus coat. This can be seen in electron micrographs. The presence of an identical enzyme, adenosinetriphosphatase, associated with both the cell membrane and the virus coat also supports this observation. The infected cell produces virus for 3 to 8 days with prolonged output of virus particles reaching a rate of 40 particles per cell per hour.

Although the presence of the virus in the myeloblast cell causes a halt in the maturation process, the cell continues its normal mitotic sequence. The concentration of the immature leukocytes builds up in the hemopoietic tissue and eventually become so great that myeloblasts are found in large quantities in the peripheral

blood. After several days, the concentration of myeloblasts is sufficiently high so that the erythrocytes can no longer carry out their respiratory function and the bird dies of acute leukemia.

LMW Virus RNA

Avian myeloblastosis (BAI strain A) virus RNA has been studied with reference to its chemical reactivity (Beaudreau et al., 1964). These experiments have shown that the ^{14}C-amino acids attached to myeloblast tRNA were apparently increased by the addition of virus RNA to the system. This was explained by the observation that the virus RNA separate from the myeloblast RNA could combine with amino acids. The binding was shown to be the result of a chemical interaction by the requirement for an energy source and by cleavage of the bond by alkaline hydrolysis. This binding activity in the virus RNA was attributed to the component that sedimented slowly on a sucrose density gradient, the low molecular weight (LMW) virus RNA. It seemed quite possible that this fraction of virus RNA could be functioning as a transfer RNA molecule.

If the virus contained a tRNA component, the infected cell could possibly be affected. The presence of additional adaptor molecules into the cell's synthetic system could upset the balance needed for normal protein synthesis. A new set of adaptors could change the whole synthetic pattern of the host in such a way as to

be more advantageous to the infecting virus. In short, the LMW virus RNA could be, at least partially, responsible for the dramatic change in the cell metabolism upon infection with the virus.

It is, therefore, the purpose of this investigation to study the biological activity of the LMW virus RNA in hopes of gaining insight into the metabolic changes observed in the infected host cell.

MATERIALS AND METHODS

Growth and Isolation of Myeloblasts

Myeloblast cells and virus particles were isolated from chicks in the terminal stage of myeloblastosis leukemia. White Leghorn chickens (Lansing Line 15 or commercial) were used. The Lansing Line was preferred for its high susceptibility to the virus.

The chicks were infected at age 3-7 days by inoculation of chick plasma containing virus particles into a wing vein. The concentration of virus particles was $5\text{-}8 \times 10^{11}$ particles per ml with 0.1 ml total inoculum. If the plasma virus concentration was too high, it was diluted with 1.5% bovin serum albumin to the desired concentration. Infection ran 75-95% in the Lansing Line 15 birds and 30-60% in the commercial lines. Nine to ten days after inoculation, the birds were bled by heart puncture, collecting as much blood as possible. The blood was aspirated from the heart into conical centrifuge tubes containing 1000 units heparin in 0.1 ml.

The tubes were then centrifuged at 1800 × g for 10 min. and the virus containing plasma supernatant removed and centrifuged again at 1800 × g for 10 min. The plasma to be used for future inoculations was labeled and stored according to the individual bird. The plasma to be used for virus RNA isolation was pooled and both were stored at -70° C. The pellet from the original centrifugation

consisted of a bottom red blood cell layer and a top white cell or myeloblast layer. The myeloblast layer was carefully suspended in a 50-50 solution of chick serum and Mixture 199 (Microbiological Associates, Bethesda, Maryland), and all the suspensions pooled. These were centrifuged at 3000 × g for 10 min., the supernatant removed and discarded, and the pelleted cells washed twice in 0.9% NaCl-0.01 M tris-mg buffer pH 7.5, and centrifuged as above. The washed cells were then stored at -20° C.

The aminoacyl-synthetase and myeloblast tRNA from the washed myeloblasts and virus RNA from the plasma were isolated from these preparations.

Myeloblast tRNA Isolation

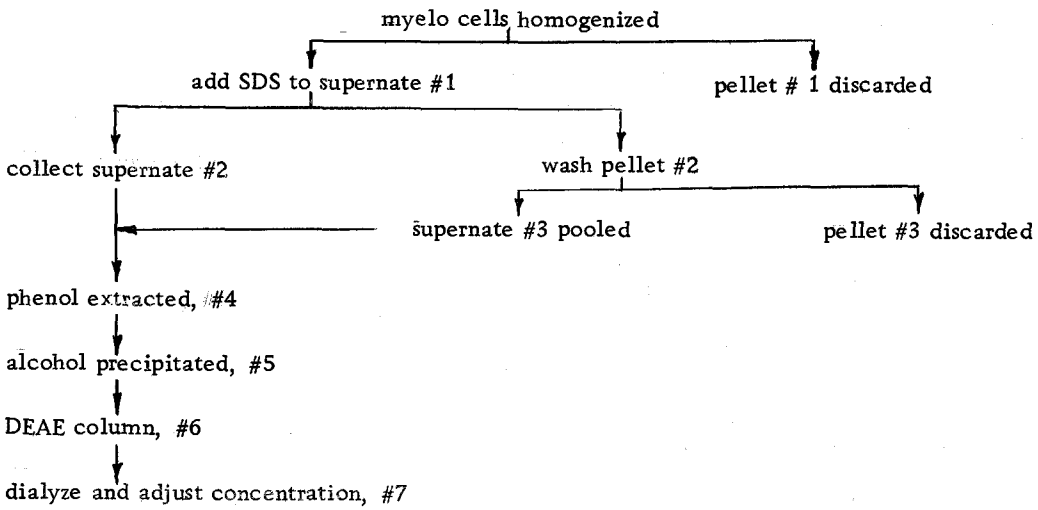

Figure 1. Schematic diagram of myeloblast tRNA isolation.

The tRNA isolation is essentially the same as Littauers as reported by Richardson (1966). Ten ml 0.1 M tris buffer pH 7.0 was added to 10 ml myeloblast cells. The cells were homogenized in ice for 1 min. at high speed setting on the VirTis homogenizer (Figure 1). The homogenized mixture was centrifuged at 20,200 × g for 45 min. The pellet (#1) was discarded and the supernate (#1) brought to a final concentration of 1% SDS (sodium dodecyl sulfate). To this an equal volume of phenol was added and allowed to shake at 4°C for 45 min. This was centrifuged at 12,100 × g for 10 min. The supernate (#2) was collected and saved and the pellet (#2) washed by adding one-half volume 0.01 M tris buffer pH 7.0 and shaking at 4°C for 15 min. After centrifugation at 12,100 × g for 10 min., the pellet (#3) was discarded and supernate #3 pooled with supernate #2. An equal volume of phenol was added to the pool (#4) and shaken at 4°C for 15 min. This was centrifuged at 12,100 × g for 10 min. and the pellet discarded. The supernate was extracted three times with an equal volume of ether and filtered.

The RNA was then alcohol precipitated (#5) by the following procedure. Two-tenths volume 2 M KAc and 2.1 volume 95% EtOH were added and allowed to stand at -20°C for 90 min. After centrifugation at 23,500 × g for 20 min., the supernate was discarded and the pellet washed with 40 ml cold 0.5 M NaCl in 67% EtOH (in 0.01 M tris pH 7.0). The RNA was centrifuged out at

12,000 × g for 10 min. The supernate was discarded and the pellet extracted three times with 5 ml portions of cold 1 M NaCl (in 0.01 M tris pH 7.0). Each time it was centrifuged at 20,200 × g for 10 min. and the supernates pooled. The pellet was finally discarded. The combined supernates were then alcohol precipitated again and washed again with 40 ml cold 0.5 M NaCl in 75% EtOH. Three 5 ml NaCl extractions followed as above with the supernates being pooled. After pooling the combined supernates the second time, the pool was allowed to stand 1 hr. at 37° C. It was cooled to 0° C and adjusted to pH 7.4. 0.3 ml 2 M KAc and 3.8 ml 95% EtOH were added, and the mixture was allowed to stand at -20° C for 30 min. The precipitated RNA was centrifuged out at 23,500 × g for 15 min. and the supernate discarded. The pellet was washed with 5 ml 0.5 M NaCl in 67% EtOH and centrifuged at 20,200 × g for 10 min. The supernate was again discarded. The pellet was dissolved in 5 ml double distilled water.

This RNA sample was then placed on a DEAE cellulose column (#6), washed with 0.2 M NaCl in 0.1 M tris pH 7.4 and eluted with 2 M NaCl in the tris buffer. The optical densities on each 5 ml fraction were determined. The peak tubes were pooled and dialyzed against 0.01 M tris pH 7.4 at 4° C overnight (#7). The sample was then concentrated by alcohol precipitation with 0.2 volume 2 M KAc and 2.1 volume cold EtOH. After standing at -20° for 1 hr., the

RNA was centrifuged out at 23, 5000 × g for 20 min. The supernate was discarded and the pellet redissolved in 2 ml 0.01 M tris buffer pH 7.4. The concentration was adjusted to 2.0 mg/ml and stored at -60° C.

A typical preparation resulted in approximately 12 mg tRNA with the 260/280 absorbancy ratio close to 2.0.

Aminoacyl-Synthetase Isolation

The aminoacyl-synthetase isolation procedure was similar to that used by Barnett (1965). Fifteen ml of myeloblast cells with 20 ml 0.1 M tris-mg buffer containing 0.006 M 2-mercapthoethanol was homogenized in ice for 1 min. at high speed on the VirTis homogenizer. The mixture was centrifuged at 23, 500 × g for 45 min. and the pellet discarded. The supernate was centrifuged at 80, 700 × g for $2\frac{1}{2}$ hours. The top two-thirds of the supernate was removed and placed on a DEAE column and the remainder discarded. The sample on the DEAE column was washed with 50 ml 0.01 M tris pH 7.5 and eluted with 50 ml 0.35 M NaCl in 0.01 M tris pH 7.5. Three ml fractions were collected and the optical densities checked. After pooling the peak tubes, 0.5 ml aliquots were taken and frozen at -70° C. The aminoacyl-synthetase used in the assays was thawed only once with 90% activity remaining after three months. Optical density readings showed a definite peak and gave 280/260 absorbancy

ratios of 1.110 to 1.385 (Figure 2). Assay indicated that the peak tube apparently contained all of the aminoacyl-synthetases, since pooling the peak tubes gave no increase in amino acid attachment to bulk tRNA using ^{14}C-amino acid mixture and allowing the reaction to go to completion.

LMW Virus RNA Isolation

Viral RNA was isolated using approximately 215 ml of plasma from the blood of infected chicks (Deeney, 1967). One gram of kieselguhr was added to the plasma and centrifuged at 1800 × g for 10 min. The plasma supernate was removed and filtered through kieselguhr on S and S black (#589) ribbon filter paper in a Buchner funnel into a chilled flask.

The virus was centrifuged out of the plasma against a gelatin pad at 75,000 × g for 60 min. The virus particles were then purified by first washing the virus layer off the gelatin pad with cold, sterile, double distilled water and thoroughly suspending it. The solution was then centrifuged at about 900 × g for 5 min., the supernate removed and the pellet suspended in water. The washing was continued until the pellet became completely resuspended. The pooled supernates were then transferred to one of the tubes containing a gelatin pad, used above, and centrifuged at 75,000 × g for 60 min. The pelleted virus was then washed and resuspended as before

in 15 ml double distilled water. SDS was added to a final concentration of 1% and allowed to stand for a few minutes to allow lysis of the suspended virus particles. The clarified solution was then centrifuged at 900 × g for 5 min. to remove any debris present.

The RNA was extracted by adding equal volume of cold water-saturated phenol containing 0.001 M EDTA and shaken for 30 min. at 0° C. After centrifugation for 10 min. at 12,000 × g the supernate was removed and iced. One-half volume of tris-KCl (0.01 M tris, 0.2 M KCl) was added to the phenol layer and shaken 15 min. in cold. This was centrifuged and the supernates pooled. To the pooled supernates one-half volume phenol was added, shaken, and centrifuged as above, two more times.

The RNA was precipitated, after extracting three times with two volumes of anhydrous ether in a separatory funnel, by adding 0.1 volume 2 M KAc and 2.1 volume 95% EtOH and allowed to stand overnight at -20° C. The precipitation was repeated once after centrifugation at 13,000 × g for 30 min. and resuspension of the pellet in tris-KCl. The precipitated RNA was again centrifuged out and suspended in 0.1 ml 0.01 M tris and layered onto a 5-20% sucrose density gradient and centrifuged at 75,000 × g for four hours. One ml fractions were collected from the gradient and the optical density at 260 mμ read. The tubes were divided into high and low molecular weight peaks, the peaks pooled, and alcohol precipitated by adding

0.1 volume 2 M KAc and 2.1 volume cold 95% EtOH and standing at -20° C until used. The low molecular weight peak is the LMW virus RNA referred to throughout this paper.

Standard Incubation Assay

The standard incubation assay was an in vitro assay to determine the amount of amino acids attached to tRNA. Each assay tube contained all the necessary materials to result in the attachment of the various ^{14}C-amino acids to their respective tRNA's. No ribosomal material was added, so that the end point was the ^{14}C-amino acid-tRNA complex.

Each assay contained the following in a 0.5 ml reaction mixture: 200 μg tRNA (in 0.01 M tris buffer pH 7.5); ATP, 5.0 μmoles; $MgCl_2 \cdot 6H_2$, 5.0 μmoles; KCl, 5.0 μmoles; 1 μc ^{14}C-amino acid mixture; 200-220 μg aminoacyl-synthetase enzyme (from DEAE column fractions in 0.01 tris buffer pH 7.5); and enough double distilled water to bring the reaction mixture to the 0.5 ml total volume. The above items were added in the following order: tRNA, Stock II (ATP, $MgCl_2$, KCl), water, ^{14}C-amino acid mixture, and enzyme. The tubes were then incubated at 33° C for one-half hour which allowed complete saturation of the tRNA available. The ^{14}C-amino acid-tRNA complex was precipitated with 10% TCA and iced for 10 min., after which the acid insoluble material was filtered

onto millipore filters using 2.5% TCA to wash any soluble, unattached ^{14}C-amino acids through the filter. The filters were then dryed and counted as described under scintillation counting procedure.

Methylated Albumin Kieselguhr Column Chromatography

The methylated albumin kieselguhr (MAK) column was prepared and used according to the procedure of Mandel and Hershey (1960). Twenty ml 0.1 MKCl in 0.05 M phosphate buffer, pH 6.7 is added to 4 g of washed kieselguhr, and 10 ml 0.1 M KCl in the same buffer is added to 1 g kieselguhr in separate beakers. The two mixtures were then boiled to expel air and cooled. One ml 1% methylated albumin was added to the 4 g mixture and stirred for 5-10 min. About one-half of the 1 g mixture was poured onto the bottom disc of the column and the 4 g mixture added on top. The column was washed with 75 ml 0.1 M KCl in the phosphate buffer. After washing, the column was ready to receive the sample.

The RNA was charged as in the standard incubation assay except that the product was not TCA precipitated. The ^{14}C-amino acid-tRNA complex was recovered by phenol extraction and alcohol precipitation. The reaction in each assay tube was halted by adding 2.5 ml 0.01 M tris buffer pH 7.0 and 6 ml phenol. This was shaken for 45 min. at 4° C. After centrifugation at 12,000 × g for 10 min., the aqueous layer was extracted three times with equal volume cold

cold ether. The tRNA was precipitated by adding 0.5 ml 2 M KAc and 12 ml cold EtOH and allowed to stand overnight at -20° C. The RNA was centrifuged out at 23, 500 × g for 20 min. The pellet was dissolved in 25 ml 0.1 M phosphate buffer pH 6.7 and placed on the column. The supernate was discarded.

The sample was washed with 50 ml 0.1 M KCl in 0.05 phosphate buffer pH 6.7 and eluted with a KCl gradient from 0.1 M to 0.8 M. A total elution volume of 200 ml KCl and a flow rate of 3 ml/min. was used. Three ml fractions were collected.

The optical density and index of refraction were first determined on each fraction. The samples were then precipitated by adding 0.3 ml 100% TCA to each tube. The acid insoluble material was filtered onto millipore filters, dryed, and the radioactivity determined on a liquid scintillation counter.

Two-Dimensional Paper Chromatography Procedure

Several methods were used to determine which tRNA's were accepting amino acids. All were based on the idea that, since the ^{14}C-amino acids were complexing with specific tRNA's, we could assay for the various tRNA's by analyzing the amino acids that were attached. The first method involved amino acid analysis by paper chromatography.

The incubation was exactly the same as described above under

the standard incubation assay procedure which resulted in a saturated tRNA solution where every tRNA molecule available was complexed with one of the ^{14}C-amino acids. The incubation was terminated by adding 2.5 volume 0.01 M tris pH 7.0, extracted using 3 ml of water-saturated phenol, shaken for 45 min. at 4° C, and centrifuged at 10,000 × g for 10 min. The water layer was extracted similarly a second time and shaken for 15 min. at 4° C. After centrifugation as above, the water layer was extracted three times with equal volume of cold ether.

The ^{14}C-amino acid-tRNA complex was then precipitated by adding 0.1 volume 2 M KAc and 2.1 volume cold EtOH and allowing it to stand overnight at -20° C. The complex was centrifuged out at 13,000 × g for 20 min. It was then precipitated a second time after being suspended in 1 ml tris pH 7.0, and allowed to stand at least three hours at -20° C. After centrifugation as above, and resuspending once again, the preparation was put into pre-boiled dialysis tubing and dialyzed against 0.01 M tris pH 7.0, overnight, in a two liter volume to remove the KAc salt.

The isolated ^{14}C-amino acid-tRNA complex was then vacuum evaporated to dryness and hydrolyzed by adding 1 ml of pH 10.5 NH_4OH to the dry preparation. This was incubated for one hour at 37° C to insure complete hydrolysis. The ammonium was then removed by vacuum evaporating to dryness.

The ^{14}C-amino acids were removed from the solution by suspending the dry material in 1 ml double distilled water, and dialyzing against 5 ml double distilled water three times, using small dialysis tubing in a conical test tube. The total 15 ml of solution from the outside of the three dialysis was then pooled and concentrated by vacuum evaporating in a 50 ml erlenmeyer flask, resuspending in 1 ml double distilled water, transferring to a conical test tube and vacuum evaporating again to dryness.

The dryed material was then suspended in 0.02 ml double distilled water and spotted onto chromatography paper. The tube was washed twice with 0.02 ml double distilled water so that a total of 0.06 ml was spotted on the paper. The chromatogram was of the two phase, descending type. The first phase consisted of 12 parts butanol, three parts acetic acid, and five parts water; and the second phase consisted of 99.5% phenol and 0.5% NH_4OH. The first phase took about seven hours for the front to move 23 cm and the second phase took three hours to move 15 cm. After drying thoroughly, the paper was exposed to X-ray film for at least two weeks. By comparison to a ninhydrin developed control, the individual amino acid spots could be identified.

Automatic Amino Acid Analysis

A second method for determining which tRNA's were accepting

amino acids was to analyze for amino acids with an automatic amino acid analyzer.

Samples for the analyzer were prepared using myeloblast tRNA and LMW virus RNA. The reaction mixture was incubated as in the standard incubation assay and the reaction terminated with 3 ml cold 0.01 M tris buffer pH 7.0. The mixture was phenol extracted with 4 ml phenol and shaken at 4° C for 45 min. The extracted material was centrifuged out at 12,100 × g for 10 min. and the supernate re-extracted with 4 ml phenol. After centrifugation at 12,100 × g for 10 min., the aqueous layer was extracted three times with equal volume of cold ether. The RNA was then precipitated by adding 0.4 ml 2 M KAc and 9 ml cold EtOH and allowing to stand at -20° C overnight. The RNA was centrifuged out at 20,200 × g for 20 min. and the supernate discarded. The pellet was dissolved in 0.01 M tris pH 7.0 and dialyzed against 1 liter 0.01 M tris pH 7.0 for 2 hrs., changing the buffer once. The RNA was then alcohol precipitated from the solution as above. The pellet was dissolved in 5 ml NH_4OH pH 10.5 and allowed to stand 1 hr. at 37° C to hydrolyze the amino acids from the tRNA. The RNA was then precipitated out with 10 ml EtOH at pH 2.0, standing 5 hrs. at -20° C. After centrifugation at 23,500 × g for 20 min., the supernate, containing the amino acids, was vacuum evaporated. One ml double distilled water was added to dissolve the evaporated amino acids. Non-labeled

amino acids were added to the ^{14}C-amino acid sample to act as an absorbancy marker since the sample was below the limit of resolution. The ^{14}C-amino acids were then determined on a Spinco Model 120B Automatic Amino Acid Analyzer[2] and compared to the absorbancy standard.

DEAE Cellulose Column Chromatography

A DEAE Cellulose (Diethyl Amino Ethyl Cellulose) Anion Exchange column, using coarse mesh with a capacity of 0.94 meq./gm in Cl^- form was used to purify RNA and enzyme and to compare the LMW virus fraction with E. coli tRNA.

About 400 ml dry DEAE Cellulose was first washed in 1 liter 1 N NaOH and the cellulose centrifuged out. It was then washed with 1 liter 1 N HCl and the cellulose again centrifuged out. Using 0.1 M tris-0.005 M Mg^{++} buffer pH 7.5, the cellulose was washed until a pH of 7.5 was reached. The cellulose was then poured into the column. The final dimensions of the column were 3 cm by 20 cm. The column was charged by washing with about 300 ml 0.1 M tris-mg buffer pH 7.5 containing 0.5 M KCl. The column was then washed with the tris-mg buffer pH 7.5 until clean. The clean column was checked by reading the index of refraction and optical density of the

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[2]Appreciation is extended to Dr. R. R. Becker for his help with the amino acid analyzer.

wash. Low readings indicated that the excess salt and extraneous material had been removed from the column.

The sample was then placed onto the washed column, followed by a sample wash and the eluent. The sample wash and the elution buffer vary in salt concentration and volume depending on the type of material that was on the column and the material to be eluded.

Liquid Scintillation Counting Procedure

The counting procedure was used to determine radioactivity of acid insoluble material filtered onto millipore filters. The filters were oven dried for 10 min. and placed into vials containing 15 ml scintillation counting fluid. The scintillation medium was made up of 4 g BBOT(2, 5-bis [2-|5-tert-butylbenzoazolyl|])-thiophene in one liter toluene. The counting vials were then cooled and placed in a Pachard Tri-Carb Liquid Scintillation Spectrometer where the radioactivity was determined. The counting efficiency of the instrument was determined using ^{3}H- and ^{14}C-toluene standards. ^{3}H counting efficiency was approximately 10% and ^{14}C counting efficiency was about 50-60%. A blank, made up of 15 ml scintillation counting fluid and a clean millipore filter in a counting vial, was used to determine background counts with each series of assays.

^{3}H-labeled and ^{14}C-labeled material could be counted at the same time on this dual channel instrument. The voltage of one

channel was set at the maximum for ^{3}H and the voltage of the other channel was set at the maximum for ^{14}C. The screening windows were then set so that approximately 10% of the ^{14}C counts appeared in the ^{3}H channel and less than 1% of the ^{3}H counts appeared in the ^{14}C channel. With this type of dual labeling, the attachment of two different amino acids could be determined at the same time with the same tRNA or LMW virus RNA.

Materials

The ^{3}H- and ^{14}C-labeled amino acids were obtained from New England Nuclear Corporation (NEN) and International Chemical and Nuclear Corporation (ICN) as noted in Table 1. All labeled amino acids, including the amino acid mixture, were adjusted to 20 μc/ml for assay use.

Table 1. Labeled amino acids.

Amino acid	Label	Specific activity μc/μmole	Source
Arginine	^{14}C	234	NEN
Serine	^{3}H	477	ICN
Valine	^{14}C	190	NEN
Threonine	^{14}C	164	NEN
Lysine	^{3}H	870	NEN
Histidine	^{14}C	222	NEN
Leucine	^{3}H	300	NEN
Alanine	^{3}H	300	NEN
Tyrosine	^{14}C	297	NEN

The ^{14}C-amino acid mixture from NEN had specific activity of approximately 40 mc/milliatom of carbon and contained the following amounts of amino acids in one mc of mixture:

L-Alanine	80 μC	L-Lysine	60 μC
L-Arginine	70 μC	L-Phenylalanine	80 μC
L-Aspartic Acid	80 μC	L-Proline	50 μC
L-Glutamic Acid	125 μC	L-Serine	40 μC
Glycine	40 μC	L-Threonine	50 μC
L-Histidine	15 μC	L-Tyrosine	40 μC
L-Isoleucine	50 μC	L-Valine	80 μC
L-Leucine	140 μC		

DEAE (Diethyl Amino Ethyl Cellulose) and the Tris buffer [Tris(hydroxymethyl) aminomethane] were obtained from Sigma Chemical Company. All other chemicals were reagent grade.

RESULTS

Beaudreau et al. (1964), have suggested that the BAI strain A (myeloblastosis) avian tumor virus contains an RNA fraction similar to transfer RNA. In order to test this hypothesis, a series of experiments were designed to characterize the virus RNA fraction as transfer RNA by demonstrating the presence of defined tRNA properties and by comparison to cellular tRNA. By definition, transfer RNA must be able to combine enzymatically with amino acids and transfer the attached amino acids to a growing peptide chain on the ribosome-mRNA complex (Watson, 1965).

Properties of Aminoacyl-Synthetase

The first reaction that tRNA enters into, the attachment of amino acids, requires the presence of the enzyme, aminoacyl-synthetase. As a preliminary to the investigations, an active enzyme preparation, free of endogenous tRNA contamination was, therefore, needed. The enzyme was isolated from infected myeloblast cells as described in the Methods section. The DEAE cellulose column used in the isolation procedure was employed primarily to remove any contaminating cellular tRNA that might be in the preparation.

The DEAE cellulose elution profile of protein (absorbancy measured at 280 mμ) is shown in Figure 2. One major protein peak

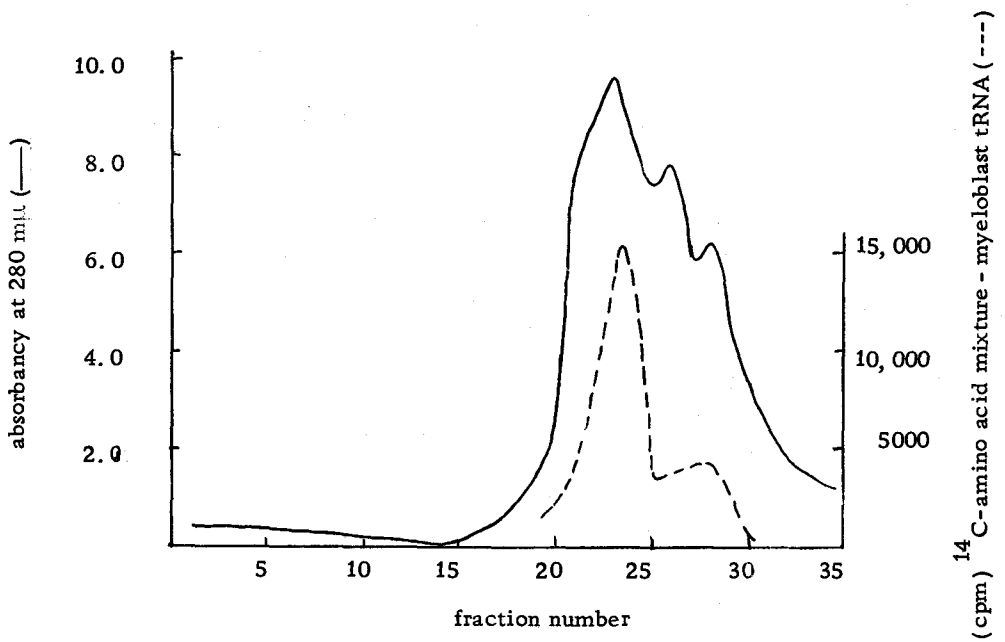

Figure 2. Elution profile of aminoacyl-synthetase on DEAE Cellulose and associated aminoacylation activity.

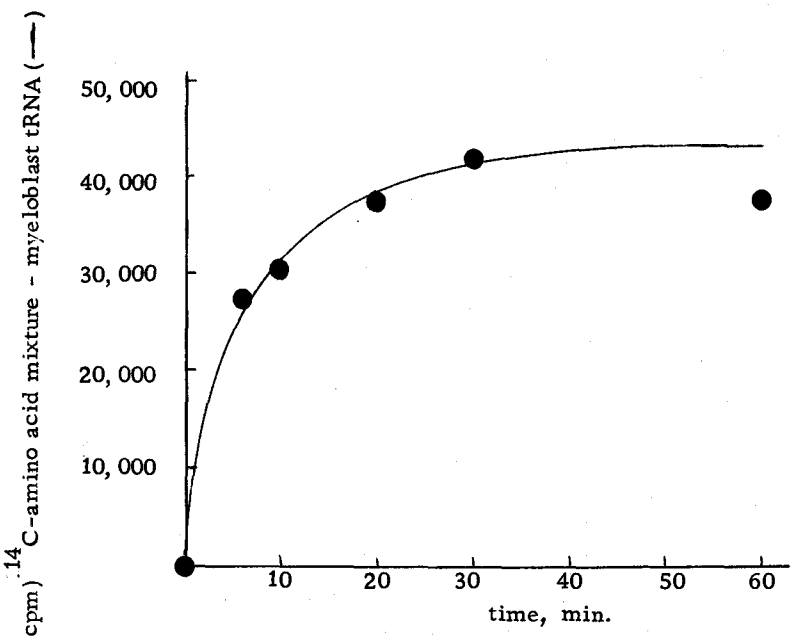

Figure 3. Time study of aminoacylation reaction.

was seen. The enzyme activity was found to be associated with the peak tubes (fractions 20-25), when tested for their ability to catalyze the amino acid attachment to myeloblast tRNA. The pooled peak tubes were shown to contain at least all of the aminoacyl-synthetases necessary for attaching the nine amino acids discussed below (Table 3).

The tRNA that may be contaminating the enzyme preparation, binds to the column more tightly and is not eluted at 0.35 M NaCl with the protein (Figure 8). This procedure reduces the endogenous tRNA to a negligible level, as shown on Table 2. The attachment of ^{14}C-amino acids attributed to the endogenous tRNA is less than 1% of the attachment seen with the addition of 200 μg myeloblast tRNA added.

The enzyme activity is shown in the kinetics study of the attachment reaction using enzyme from the pooled peak tubes (Figure 3). With 200 μg myeloblast tRNA, saturation of the tRNA by amino acids occurred at approximately 30 min. incubation time at 35° C under the same assay conditions as described in the Methods section.

The aminoacyl-synthetase in the pooled fractions 20-25 met the requirements of activity and purity and was used in the experiments throughout the investigation.

Properties of the Virus RNA

The LMW virus RNA fraction is separated from a high molecular weight fraction on a 5-20% sucrose density gradient (Figure 4). The LMW peak has a sedimentation coefficient of about 4 S as compared to 64 S for the high molecular weight peak. The 64 S material is thought to be the viral genome.

The first step in characterizing the LMW virus RNA fraction as transfer RNA was to compare the chromatographic properties of the LMW virus RNA fraction with cellular tRNA. In a strict sense, tRNA's of different origin need not all have the same chromatographic properties. However, all tRNA's appear to contain about the same number of bases and have similar secondary structure and, therefore, might be expected to be chromatographically very similar. A similarity between the LMW virus RNA and cellular tRNA would strengthen the hypothesis that the virus fraction is tRNA.

LMW virus RNA and myeloblast tRNA were first compared on a methylated albumin kieselguhr (MAK) column. The MAK column separates molecules on the basis of size, structure, and base composition. The virus RNA fraction was charged with a mixture of ^{14}C-amino acids and the myeloblast tRNA was charged with ^{3}H-lysine. The two reactions were incubated separately under identical conditions

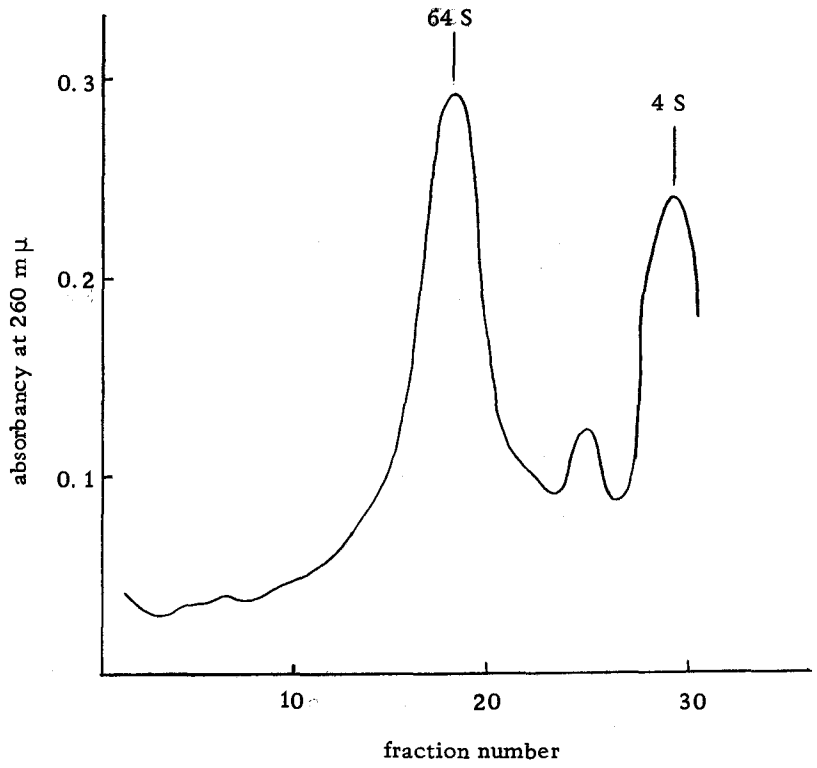

Figure 4. Fractionation of myeloblastosis virus RNA by sucrose density gradient centrifugation. Myeloblastosis virus RNA was layered on top of a 5-20% sucrose gradient (sucrose solution was in 0.01 M tris buffer, pH 7.8, 0.1 M NaCl, 0.001 M EDTA) and centrifuged in a SW 25.1 rotor, Spinco Model L, for four hours at 25,000 rev./min.

(as described in Methods, standard incubation assay). After terminating each reaction mixture by dilution with four volumes of cold buffer, the samples were combined and the RNA isolated. The amino acid-tRNA complexes were then chromatographed on a MAK column. The fractions were scanned for absorbancy at 260 mμ and for radioactivity. Radioactivity in a fraction would indicate the presence of the amino acid-tRNA complex. The dual label procedure is particularly suited for this experiment, since both samples can be chromatographed on the same column, thus eliminating the possible error introduced by comparing two separate columns. Figure 5 shows the results of this experiment.

The ^{3}H-lysyl-tRNA was eluted at the same KCl concentration (0.5 M) as was the ^{14}C-amino acid-virus RNA complex. These results demonstrate that amino acids are being attached to RNA molecules from the LMW virus fraction that have size and structure properties similar to myeloblast tRNA. The results in Figure 6 indicate that the LMW virus RNA can actually be broken into two subfractions. In this experiment only the ^{14}C-amino acid-virus RNA complex was chromatographed. One of the absorbancy peaks (measured at 260 mμ) has ^{14}C-amino acids associated with the RNA. The other absorbancy peak is devoid of associated ^{14}C-amino acids. This pattern of subfractions was characteristic of all LMW virus RNA fractions that were tested. This subfraction showing no

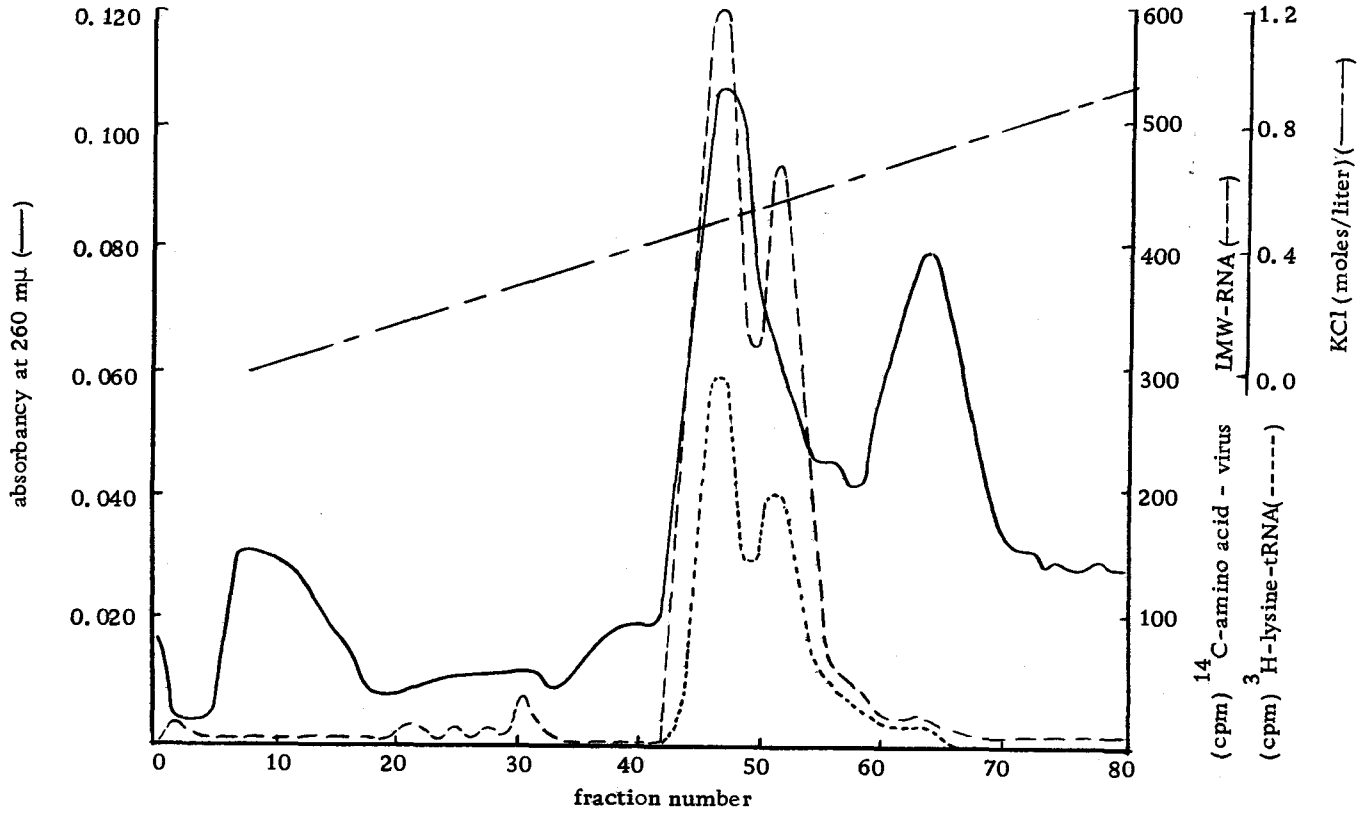

Figure 5. MAK column analysis of LMW virus RNA fraction and cell tRNA. 225 μg of LMW virus RNA, charged with ^{14}C-amino acid mixture, and 200 μg myeloblast tRNA, charged with ^{3}H-lysine, were applied to the column. The elution gradient ran from 0.1-0.8 M KCl in 0.05 M phosphate buffer pH 6.7.

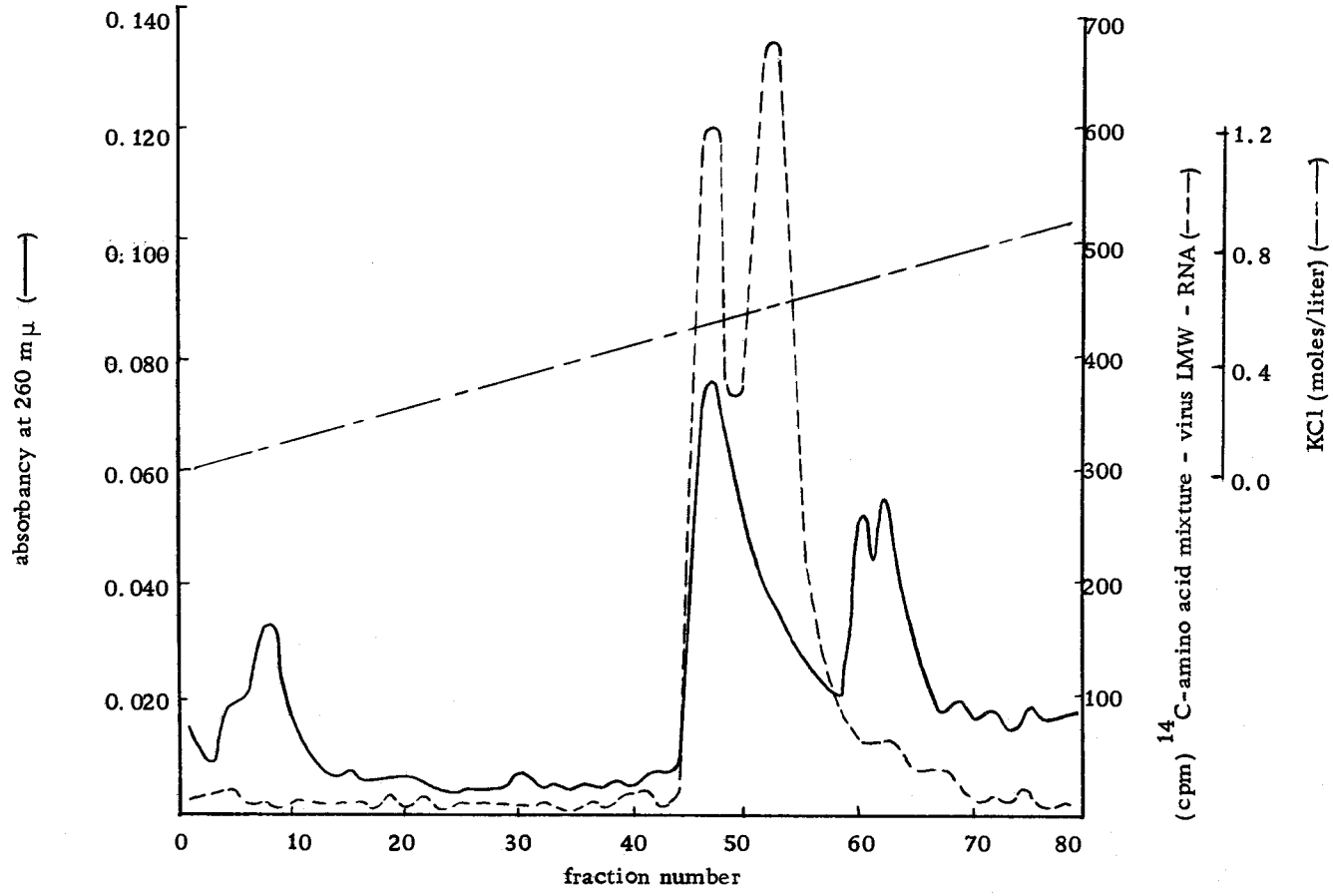

Figure 6. MAK column analysis of ^{14}C-amino acid-LMW virus RNA complex. 200 μg LMW virus RNA, charged with ^{14}C-amino acid mixture was applied to column under the same conditions used for the experiment described in Figure 5.

associated ^{14}C-amino acids is also seen in Figure 5.

For comparison with previous experiments the myeloblast tRNA charged with ^{14}C-amino acids was also chromatographed on a separate MAK column. It can be seen in Figure 7, that cellular tRNA is eluded at 0.5 M KCl, which is the same salt concentration observed for the amino acid-RNA complexes in Figures 5 and 6. However, no absorbancy peak is seen in the 0.7 M KCl region, further indicating that the RNA peak appearing in that region in Figures 5 and 6 is indeed from the LMW virus RNA fraction.

These MAK column data, therefore, show that a portion of the LMW virus RNA does compare favorably with myeloblast tRNA.

One additional interesting feature can be seen in Figures 5 and 6. The RNA eluding at 0.5 M KCl has two minor peaks. Lysyl-tRNA is known to exist in two forms in E. coli (Sueko and Yamane, 1962). The two small ^{3}H-lysyl tRNA peaks seen in Figure 5 might also be due to the presence of two lysine specific tRNA's in the myeloblast cell. It is surprising, however, to have the absorbancy peak and the ^{14}C-amino acid peak also show two minor peaks, since these samples are made up of all of the various tRNA species. E. coli tRNA does have a tendency to separate into two groups on a MAK column (Kano-Sueko and Sueoka, 1966). It is possible that the RNA species of the virus also tend to separate into two groups on the MAK column.

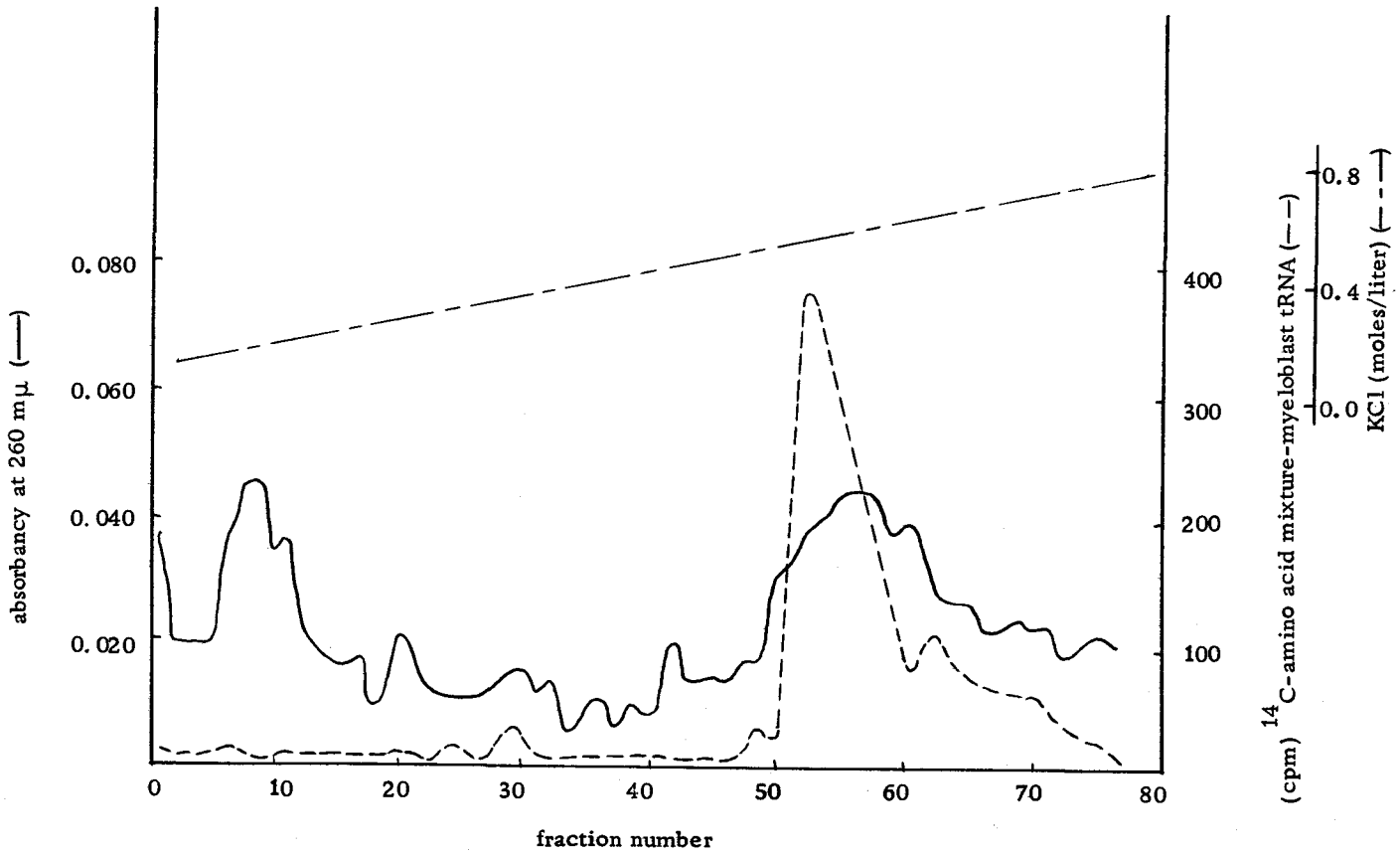

Figure 7. MAK column analysis of ^{14}C-amino acid-myeloblast tRNA complex. 200 μg myeloblast tRNA, charged with ^{14}C-amino acid mixture was applied to column under the same conditions used for the experiment described in Figure 5.

The next step in characterizing the virus RNA was to compare the DEAE cellulose column chromatography of LMW virus RNA and E. coli tRNA. The DEAE cellulose column is an anion exchange column which will bind RNA. However, only tRNA can be eluted with relatively low salt concentrations. Even fragments consisting of only 3-4 bases require extreme conditions for elution (Staehelin, 1963). The ease of eluting tRNA is probably associated with its secondary structure. Therefore, it was felt that if the virus RNA could be eluted under the same conditions as cellular tRNA, this data would provide a strong evidence for tRNA in the LMW virus fraction.

For this experiment, the virus was purified by the usual procedure and passed through as agarose column (5M BioRad 100-200 mesh). The virus was lysed with SDS and the high molecular weight RNA separated from the LMW RNA by centrifugation in a sucrose density gradient. The LMW RNA fraction contained virus protein and was purified by phenol extraction. The LMW virus RNA fraction was 20% of the total virus RNA. Figure 8 shows the absorbancy profile (at 260 mμ) for LMW virus RNA and E. coli tRNA chromatographed on DEAE cellulose under identical conditions. Both are seen to elute at about the same salt concentration, but only 43% of the input LMW virus RNA was eluted in the tRNA region. As shown in Table 2, this portion contained RNA capable of

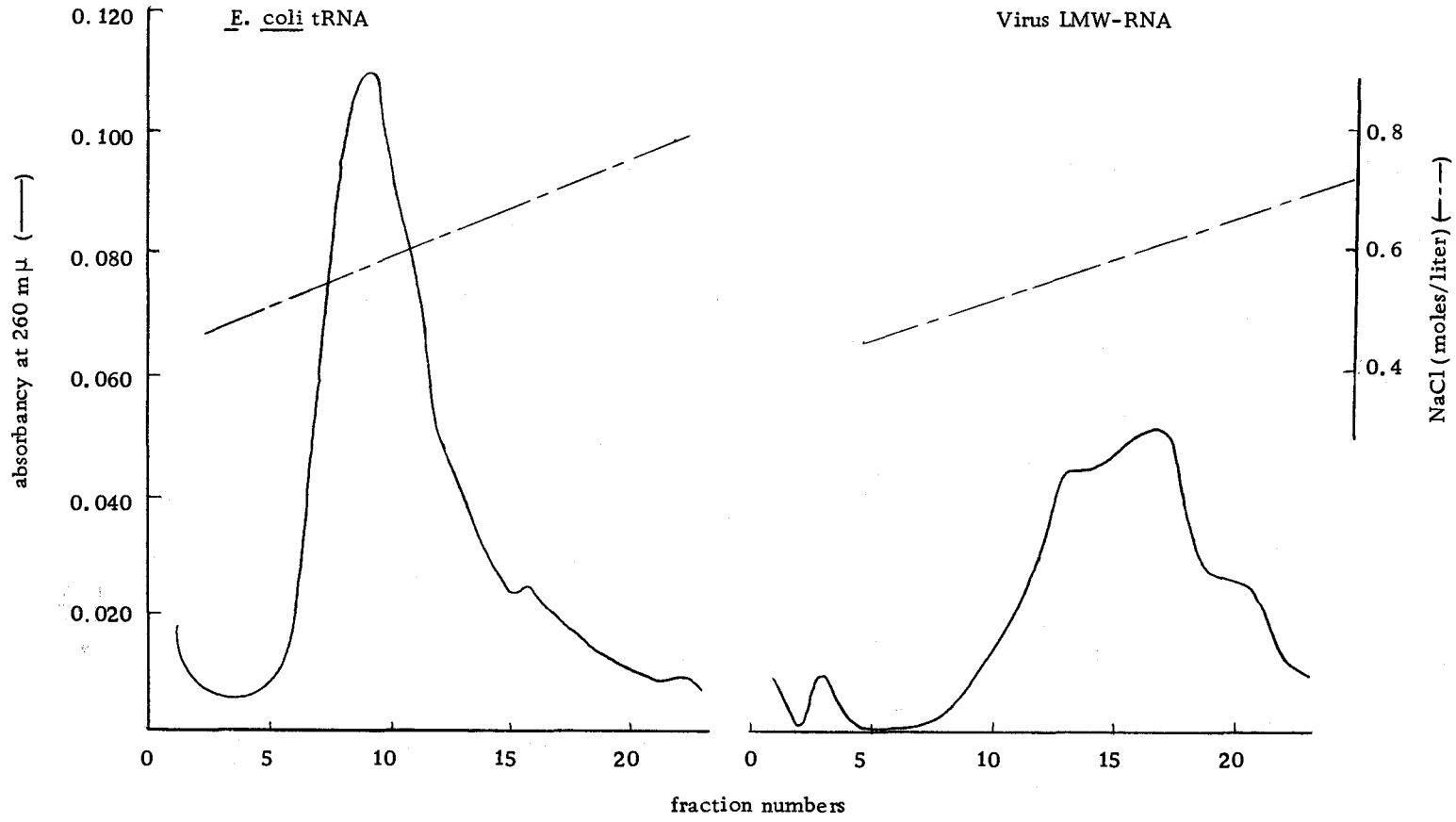

Figure 8. Chromatography of LMW virus RNA fraction and E. coli tRNA on DEAE cellulose column. (A) 200 μg E. coli tRNA and (B) 135 μg LMW virus RNA were analyzed. The elution gradient ran from 0.4-1.0 M NaCl in 0.01 M tris buffer, pH 7.0 in both cases.

accepting amino acids. The remainder of the RNA was bound tightly to the column and could only be removed after alkaline hydrolysis. There probably is some correlation between this tightly bound portion and the virus RNA peak in the 0.7 M KCl region on the MAK column in Figure 5. It is not unreasonable to believe that both of these fractions may contain fragmented viral RNA.

Since only 43% of the input LMW virus RNA eluted and the LMW fraction was 20% of the total virus RNA, only 8-9% of the total virus RNA can be said to have properties congruent with tRNA. This is consistent with data recently published by Bonar et al. (1967), which suggests that 7% of the total virus RNA has acceptance ability.

Amino Acid Acceptance Activity of LMW Virus RNA

Having demonstrated that at least a portion of the LMW virus RNA had physical properties similar to cellular tRNA, it was next essential to show that the fraction had the capacity to carry out the two biochemical reactions required of tRNA by definition.

The first of these reactions is to combine enzymatically with amino acids. The LMW virus RNA fraction from the DEAE cellulose column (Figure 8) was recovered and first examined for its ability to accept a mixture of ^{14}C-amino acids. The standard incubation assay procedure was used. The in vitro reaction in this assay

procedure was shown to be a chemical interaction by the requirement for an energy source and by cleavage of the amino acid-RNA bond by weak alkali (Beaudreau et al., 1964). The data showing the formation of amino-acid-virus RNA complexes are presented in Table 2. As can be seen, the ^{14}C-amino acid acceptance by virus RNA is similar to the acceptance by tRNA isolated from myeloblasts. The blank, containing no added RNA, is less than 1% of the myeloblast tRNA activity and shows that the endogenous RNA contamination of the enzyme preparation is negligible. With no RNA contaminating the aminoacyl-synthetase, the total radioactivity measured can be attributed to ^{14}C-amino acid attachment to the RNA added. These results are characteristic of other similar experiments and have been duplicated many times.

Table 2. Attachment of ^{14}C-amino acid mixture to the LMW virus RNA after purification on DEAE cellulose column.

RNA	^{14}C-amino acids attached to RNA (CPM $\times 10^{-3}$)	Specific activity (CPM/μg RNA)
Myeloblast tRNA (200 μg)	75.2	376
Virus RNA* (40 μg)	13.0	325
None	0.3	---

*LMW virus RNA that was removed by 0.5-0.8 M NaCl. The elution profile is shown in Figure 8.

Having shown that the LMW virus RNA can accept amino acids comparable to cellular tRNA, it was of interest to study the acceptance behavior of the LMW virus fraction as exhibited toward single amino acids. Not all tRNA species are present in a cell in the same concentrations. This is reasonable since not all amino acids are needed to the same extent in protein synthesis. Does the concentration pattern found in the LMW virus RNA fraction reflect that for tRNA in the myeloblast? An answer to this question could shed light on two important questions. What is the origin of the LMW virus RNA and what is its function?

In these experiments the standard incubation assay is used with only a single labeled amino acid added to the reaction mixture. Table 3 compares the acceptance of nine amino acids by the LMW virus RNA fraction with the acceptance by tRNA from infected myeloblasts. The comparison is made on the basis of μmoles of amino acids accepted per 100 μg RNA, to compensate for different amounts of RNA added and different specific activities among the labeled amino acids.

The attachment to myeloblast tRNA by all nine amino acids was good, ranging from 4.4-32.2 μμmoles/100 μg RNA. The fact that all nine amino acids do show good acceptance with myeloblast tRNA shows that the specific aminoacyl-synthetases for all nine amino acids are present in the enzyme preparation.

Table 3. Attachment of single amino acids to cell and virus RNA.

Amino acid	Enzyme blank ($\mu\mu$moles)	Amino acid attachment * myeloblast tRNA ($\mu\mu$ moles/100 μg RNA)	virus RNA** ($\mu\mu$ moles/100 μg RNA)	Relative activity +
^{14}C-arginine	0.21	27.2	34.0	1.25
^{3}H-serine	3.34	8.3	14.2	1.71
^{14}C-valine	0.14	4.4	0.3	0.07
^{14}C-threonine	1.03	25.6	11.6	0.45
^{3}H-lysine	1.89	32.2	2.9	0.09
^{14}C-histidine	0.02	25.3	6.9	0.27
^{3}H-leucine	25.80	14.1	11.5	0.82
^{3}H-alanine	2.06	4.5	2.6	0.58
^{14}C-tyrosine	5.26	15.2	4.8	0.32

*Enzyme blanks were subtrated from the reported values.
**Low molecular weight fraction from the virus RNA.
+ $\mu\mu$moles of amino acid attached to virus RNA/$\mu\mu$moles of amino acid attached to myeloblast tRNA.

The acceptance of the amino acids to LMW virus RNA ranges from 0.3-34.0 $\mu\mu$moles/100 μg RNA. Valine and lysine are essentially not accepted. The values for valine and lysine are considered to be at the limit of detection in this assay. On the other hand, arginine and serine are accepted more readily by the virus RNA than by myeloblast tRNA.

Two-dimensional paper chromatography and automatic amino

acid analysis were used to verify the results presented in Table 3. These two methods were employed to analyze amino acids that had been hydrolyzed from the ^{14}C- and ^{3}H-amino acid-RNA complexes (Table 4).

No data was obtained from the paper chromatography for amino acid attachment to LMW virus RNA, since a prohibitive amount of virus RNA would have to have been used to observe the amino acid spots. The paper chromatography does, however, verify the acceptance of all nine amino acids by myeloblast tRNA. The three basic amino acids, arginine, lysine, and histidine, were not monitored on the long column of the automatic amino acid analyzer. The other six amino acids were again shown to be accepted by myeloblast tRNA. Amino acid acceptance by LMW virus RNA was shown to be lacking for valine and tyrosine and serine produced a questionable result. A relatively high background on the analyzer prohibited any quantitative analysis of the acceptance.

Unfortunately, due to the scarcity of LMW virus RNA, only the acceptance assay of ^{14}C-valine in Table 3 was repeated, and the experiments were found to be in agreement. It would be desirable to test more of the amino acids and to repeat those already studied. However in each instance the LMW RNA reactions were monitored by parallel myeloblast tRNA reactions. The consistency observed in the myeloblast reactions, supports the reliability of the data

Table 4. Summary of amino acid accepting ability of low molecular weight virus RNA and myeloblast cell tRNA.

Amino acid	Single amino acid acceptance		Automatic amino acid analyzer		Two-dimensional paper chromatography	
	Virus RNA	Cell tRNA	Virus RNA	Cell tRNA	Virus RNA	Cell tRNA
Arginine	+	+	0	0	0	?
Serine	+	+	?	+	0	+
Valine	-	+	-	+	0	+
Threonine	+	+	+	+	0	+
Lysine	-	+	0	0	0	+
Histidine	?	+	0	0	0	?
Leucine	+	+	+	+	0	?
Alanine	+	+	+	+	0	+
Tyrosine	?	+	-	+	0	+

0 Indicates no data collected
+ Indicates amino acid accepted by RNA
- Indicates amino acid not accepted by RNA
? Indicates amino acid acceptance questionable

presented for the LMW virus reactions. It can be said, with reasonable confidence, that there definitely is a different amino acid acceptance pattern exhibited by the LMW fraction than is seen in the myeloblast tRNA. The bearing that this has on the two questions posed earlier will be discussed in later sections.

Transfer Activity of the LMW Virus RNA

The second biochemical reaction required of tRNA is the transfer of the attached amino acid to a growing peptide chain.

There is an RNA component in the LMW virus RNA fraction that inhibits peptide synthesis using a cell-free E. coli system (Olsen, Deeney, and Beaudreau, in press). Consequently, this system could not be used to study the transfer process. However, these investigators have shown that, fortunately, inhibition of peptide synthesis by the LMW RNA fraction occurs prior to attachment of the mRNA to the ribosome. It was found that a cell-free polysome system from virus-infected myeloblasts would form peptides in the presence of the LMW virus RNA.

The standard polysome system contains energy-generating components, amino acids (one of which is labeled), a ribosome fraction, and a pH 5 enzyme active fraction that also contains tRNA. Figure 9 shows the kinetics of the myeloblast polysome system. In this experiment 15.2 mg/ml ribosomes and 33.0 mg/ml pH 5

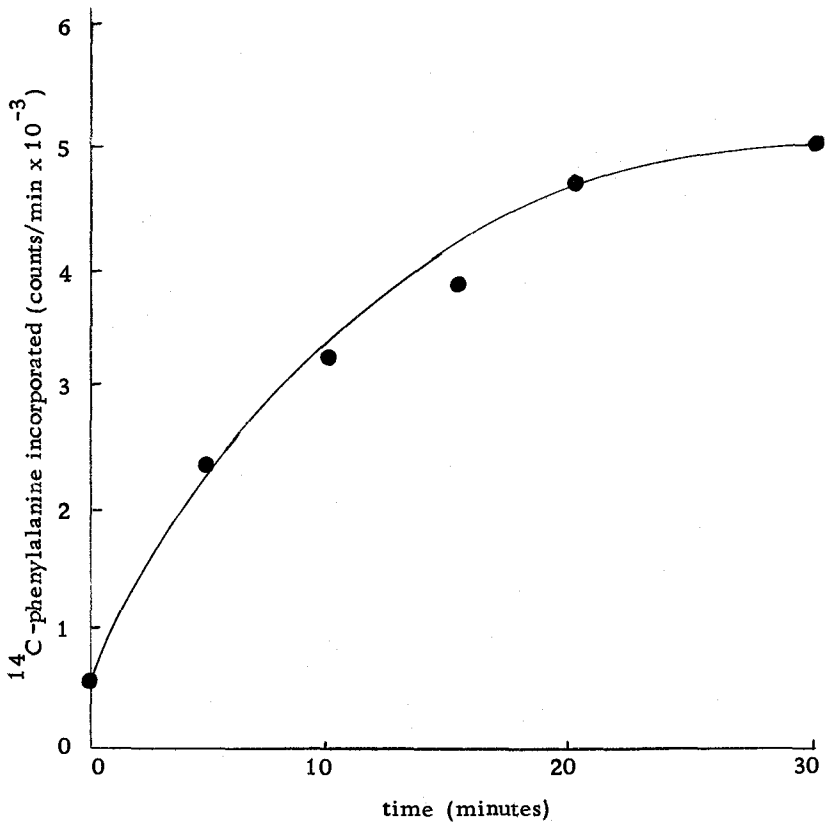

Figure 9. Kinetics of polysome fraction. The reaction contained 6mM phosphoenolpyruvate, 50 μg/ml pyruvate kinase, 1.5mM ATP, 0.3mM GTP, 10mM magnesium acetate, 30mM KCl, 40mM Tris-HCl pH 7.6, 8mM 2-mercapthoethanol, 4×10^{-5} M amino acids (minus phenylalanine), 0.16 μC ^{14}C-phenylalanine, 15.2 mg/ml ribosome fraction, and 33 g/ml pH 5 enzyme in a total volume of 0.10 ml.

enzyme were incubated for varying lengths of time with the standard energy-generating components, cold amino acids (minus phenylalanine), and ^{14}C-phenylalanine in a total volume of 0.1 ml. After incubation the reaction mixture was treated with hot TCA to hydrolyze the ester linkages between the tRNA and amino acids and precipitate the newly formed peptides. The insoluble material was then filtered out onto a millipore filter and the radioactivity on the millipore filter determined on a liquid scintillation counter. The ^{14}C-phenylalanine counted was part of the new peptide and would be a relative measure of overall peptide synthesis. As Figure 9 shows, synthesis increases as the incubation time increases.

This active polysome system was then used to further characterize the LMW virus RNA as a tRNA by demonstrating the presence of transfer activity in the LMW fraction. LMW virus RNA was charged with ^{14}C-amino acid mixture by the standard incubation assay procedure. The ^{14}C-amino acid-virus RNA complex was then isolated and incubated in the complete chick cell-free polysome system.

About the same percentage of ^{14}C-amino acids was transferred to peptides from the cell tRNA and the LMW virus RNA (Table 5). When the cell-free polysome system was excluded (line 3 and 4 in table), the ^{14}C-amino acids were cleaved from the RNA by the hot TCA used to terminate the reaction. This provides strong evidence

that the RNA associated with the virus contains two of the recognition sites of tRNA. The close physical similarities to cellular tRNA and the amino acid acceptance and transfer ability leave little doubt that a portion of the RNA isolated from the virus is indeed tRNA.

Table 5. Transfer of amino acids from the LMW virus RNA to peptides.

Source of ^{14}C-aminoacyl RNA	Cell-free * polysome system	Transfer to peptides (CPM)	% transfer
Virus (1088 CPM)	+	634	58
Cell (2030 CPM)	+	969	47
Virus (1088 CPM)	-	6	--
Cell (2030 CPM)	-	9	--

*The cell-free polysome system consists of myeloblast polysomes and dialysed supernatant enzyme from chick liver. Polysomes were obtained from virus-infected myeloblasts by the technique described by Wettstein et al. (1963). 0.15 mg of polysomes was added to each reaction mixture. Dialysed supernatant enzymes were prepared by centrifuging a liver homogenate 105,000 × g for three hours and dialysing the supernatant fraction for 14 hr. The optimal concentration of 0.63 mg was added to the reaction mixture. The reaction mixture was similar to that previously used (Olson, Deeney and Beaudreau, in press). When 0.4 μc of ^{14}C-amino acid mixture and 20 μg of liver tRNA were used in this cell-free system, 24,000 cpm were incorporated into peptides in 30 min at 35° C.

Source of the LMW Virus RNA

The presence of tRNA in the virus is unusual and speculation as to its origin is interesting. There are several observations to be made regarding the source of the LMW virus RNA.

One possibility is that the LMW virus is actually cellular tRNA

adsorbing to the virus particle and being isolated along with the virus RNA. In order to exclude this possibility, 104 μg of ^{3}H-tRNA (1,400 cpm/μg) were added to 225 ml of plasma containing virus. If RNA is adsorbing onto the virus particle and not removed during the isolation process, the ^{3}H radioactivity should be present in the final virus RNA fraction. The virus was purified from the plasma and the RNA extracted from the virus. Less than 0.07 μg of ^{3}H-tRNA was actually observed in the final virus RNA fraction (Figure 10 and Table 6). This is a negligible amount compared to the total amount of virus RNA isolated. Similar results were reported by others using radioactive labeled cell RNA (Bonar et al., 1967). These experiments exclude any possibility of surface contamination by an extraviral source of tRNA, which means the LMW virus RNA fraction does exist within the virus particle.

It is possible to separate the LMW virus RNA fraction from fragmented high molecular weight virus RNA on a MAK column (Figure 11). The virus RNA applied to the MAK in this experiment had previously been shown by sucrose density gradient centrifugation to be fragmented virus RNA. As can be seen in Figure 11, a large peak eluding under higher salt concentration is present in addition to the normal profile for virus RNA (as in Figure 6). Since the LMW fraction is not entirely fragmented high molecular weight virus RNA, some of the LMW RNA must originate in the cell before

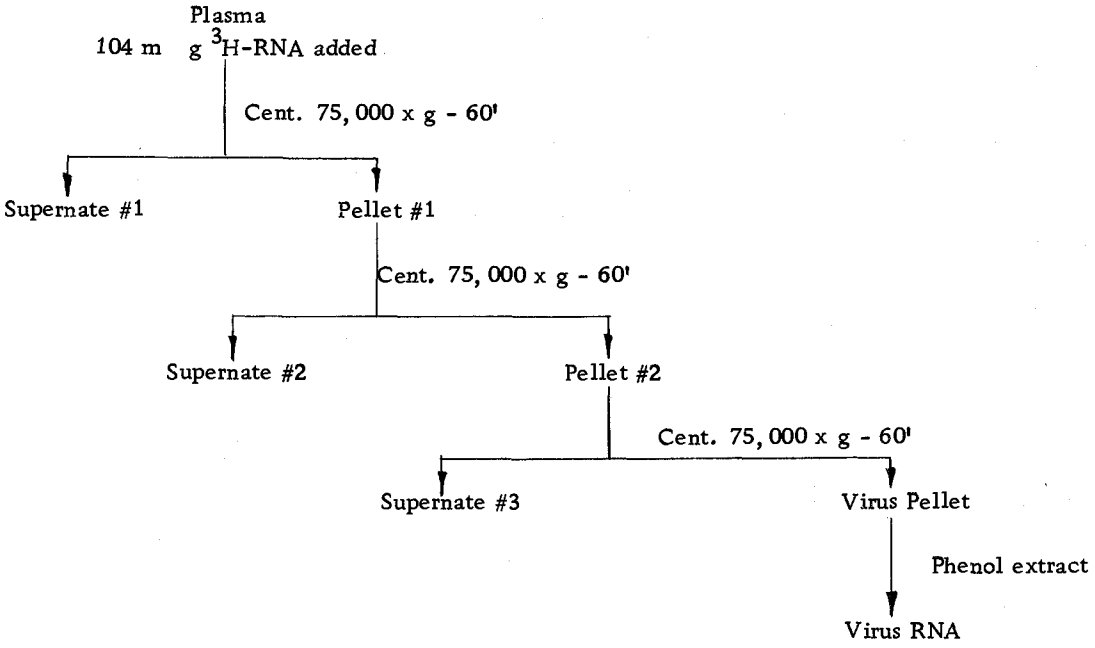

Figure 10. Schematic diagram of virus RNA isolation to determine extraviral RNA contamination.

Table 6. Extraviral RNA contaminating virus RNA preparation.

Sample (from Figure 10)	total cpm	μg ^{3}H-RNA
Plasma	146, 000	104
Supernate #1	108, 000	77
Virus Pellet	195	0. 14
Virus RNA	80	0. 06

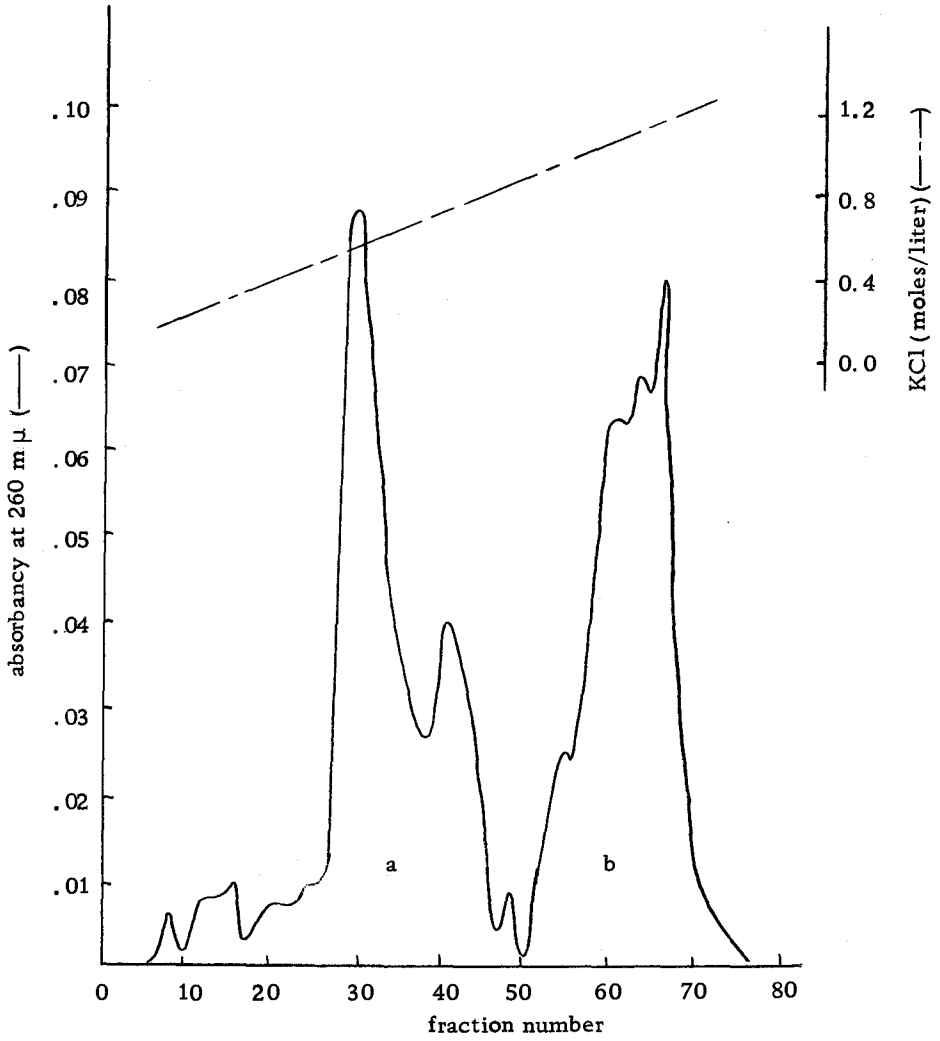

Figure 11. MAK column analysis of fragmented virus RNA preparation. Virus RNA, fragmented during phenol extraction, was applied to the column and eluted with a 0.2-1.2 M KCl gradient in 0.05 M phosphate buffer pH 6.7.

entering the virus particle. Experiments of Sverak (1962) and Bauer (1966) indicated RNA of cellular origin is contained in the virus. Since the virus particle is formed by budding from the host cell, it is possible that it could incorporate cellular tRNA inside the viral coat. If this were the case, one would expect to see a distribution of tRNA's that reflect the distribution of the host cell tRNA's. However, the broad range of tRNA activity exhibited by the viral RNA fraction toward the nine amino acids tested suggests that the process of incorporation of tRNA into the virus particle is not random trapping.

Another possibility exists that does not contradict these observations. The LMW virus RNA molecules could be transcribed off of the viral genome (high molecular weight fraction) while still inside the host cell prior to liberation. In this case the LMW fraction would be of cellular origin but genetically different from the tRNA transcribed from cell DNA. The possibility could be checked by hybridization studies of the LMW virus RNA fraction with cell DNA and with the viral genome.

SUMMARY AND CONCLUSIONS

A fraction of the RNA from avian myeloblastosis virus was studied with particular reference to its similarity to transfer RNA. The bulk viral RNA can be fractionated by centrifugation on a sucrose density gradient into two major peaks. One of these, the slower sedimenting fraction, has a sedimentation coefficient of 4S and is referred to as low molecular weight (LMW) virus RNA.

The 4S material (LMW virus RNA) has physical properties similar to tRNA from E. coli and myeloblast cells. E. coli and LMW virus RNA chromatograph the same on a DEAE cellulose column. When low virus RNA is chromatographed on a MAK column, two RNA subfractions are observed; one corresponding to myeloblast tRNA and the other characteristic of the LMW virus RNA only. Only the subfraction eluting in a manner similar to myeloblast tRNA has the ability to enzymatically accept amino acids. This portion of LMW virus RNA is found to be about 8-9% of the total viral RNA.

The LMW virus RNA accepts amino acids in a aminoacyl-synthetase catalyzed reaction to the same extent as the same amount of myeloblast tRNA. In addition, the amino acids attached to the LMW virus RNA in such a reaction, can be transferred to a growing peptide chain in a cell-free polysome system. These experiments leave little doubt that the LMW virus RNA contains an RNA fraction

that has the properties of tRNA.

The attachment activity of the LMW virus RNA for single amino acids is found to vary considerably from the attachment activity exhibited by myeloblast tRNA for the same single amino acids. Valine and lysine are essentially not attached by the LMW virus RNA while arginine and serine are attached to a greater degree by the LMW virus RNA than by the myeloblast tRNA.

The RNA was shown to be free of contamination by an extra-viral source of tRNA. The LMW fraction was also shown to contain molecules other than high molecular weight virus RNA fragments. The broad range of tRNA activity exhibited by the LMW virus RNA toward the single amino acids strongly indicates that the tRNA incorporated into the virus particle during the liberation process is not by random trapping.

Modifications of tRNA's have been shown to occur during metabolic changes such as sporulation (Doi and Kaneko, 1966) and phage infection (Kano-Sueoka, 1966), suggesting that they may be involved in translational control. The difference observed in the amino acid acceptance patterns in myeloblast tRNA and LMW virus RNA may also be significant in terms of translational control. The presence of transfer RNA in a tumor virus is of interest since the oncogenic property of the virus implies a mechanism of continuous translational control in the host cell. It is possible that the LMW

virus RNA's, having a different amino acid acceptance pattern, build up in the cell to a point where the normal cellular protein synthesis is impaired sufficiently to halt the maturation process of the host cell. Virus development could be highly favored under these conditions. Exactly what the inhibition mechanism may be is yet to be determined. Studies should be made on the myeloblast cell protein synthesis system. If a normal myeloblast system could be developed, the inhibitory action of the LMW virus fraction could then be studied at the transcriptional and translational levels.

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