

To the Graduate Council:

I am submitting herewith a dissertation written by Clinton D. Lothrop Jr. entitled "RNA Catabolism in Cultured Cells: Modified Nucleoside Excretion, Effects of RNA Synthesis Inhibitors on RNA Metabolism, and the Origin of Excreted Modified Nucleosides." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

Mayo Uziel  
Mayo Uziel, Major Professor

We have read this dissertation  
and recommend its acceptance:

E. H. Grell

Sankar Mitra

E. Uziel

Accepted for the Council:

L. Evans  
Vice Chancellor  
Graduate Studies and Research

RNA CATABOLISM IN CULTURED CELLS: MODIFIED NUCLEOSIDE  
EXCRETION, EFFECTS OF RNA SYNTHESIS INHIBITORS  
ON RNA METABOLISM, AND THE ORIGIN OF EXCRETED  
MODIFIED NUCLEOSIDES

A Dissertation  
Presented for the  
Doctor of Philosophy  
Degree  
The University of Tennessee, Knoxville

Clinton D. Lothrop Jr.

March 1981

**3049556**

## ACKNOWLEDGMENTS

I would like to thank my committee members Ed Grell, Ken Volkin, and Sankar Mitra for their knowledge and guidance. I especially wish to express my appreciation to my major professor, Mayo Uziel, for his patience and wisdom to allow me to pursue my own directions. Finally, I wish to acknowledge my wife, Kim, and my daughter, Kari, who make it all worth-while.

## ABSTRACT

The aims of this dissertation are: To determine what methylated nucleosides are excreted by cultured cells; to determine the excretion rates of these compounds; and to determine the origin of excreted modified nucleosides.

These studies are facilitated by a new rapid procedure for purifying nucleic acid components from biological fluids based on batch adsorption and elution from commercially prepaced silica cartridges. In this procedure nucleic acid components are separated into three groups (bases, deoxy and 2'-O-methyl nucleosides; nucleosides; and charged nucleosides). This fractionation is a necessary preparative step prior to identification of the nucleic acid components on an analytical scale.

I have shown that cultured fibroblasts, do indeed, excrete modified nucleosides into the culture media. A number of the excreted base and ribose methylated nucleosides were chemically identified. For the most part, the modified components of RNA are liberated as nucleosides during catabolism and not further metabolized. However, there are several exceptions. First, the methylated nucleoside 7-MeGuo, is not excreted as a nucleoside but rather as the base, 7-MeGua, indicating cleavage of the glycosidic bond. Second, 2'-O-MeAdo, a major ribose methylated nucleoside in tRNA, as well as rRNA, is not excreted. However, 2'-O-MeIno is excreted even though it is not present in RNAs in significant levels. One possible source of 2'-O-MeIno is deamination of 2'-MeAdo. Third, the base methylated nucleoside, ribothymidine,

can be salvaged by the fibroblast cells and converted to deoxythymidylate for DNA synthesis. All other methylated nucleosides are excreted without modification or intracellular accumulation. Ribothymidine is also excreted.

Finally, and most importantly, I have shown that excreted methylated nucleosides are derived from the turnover of rRNA and tRNA, not only tRNA as was previously thought. This opens the possibility that the increased excretion of modified nucleosides in cancer patients might not be from increased tRNA turnover, as previously suggested, but could result from changes in rRNA as well as tRNA metabolism.

## TABLE OF CONTENTS

| CHAPTER                               | PAGE |
|---------------------------------------|------|
| I. INTRODUCTION . . . . .             | 1    |
| II. EXPERIMENTAL PROCEDURES . . . . . | 9    |
| III. RESULTS AND DISCUSSION . . . . . | 22   |
| BIBLIOGRAPHY . . . . .                | 78   |
| APPENDIXES . . . . .                  | 85   |
| APPENDIX A . . . . .                  | 86   |
| APPENDIX B . . . . .                  | 101  |
| VITA . . . . .                        | 115  |

# LIST OF TABLES

| TABLE                                                                                                                                  | PAGE |
|----------------------------------------------------------------------------------------------------------------------------------------|------|
| 1. Separation of Excreted Methylated Nucleosides by Cation-Exchange Chromatography . . . . .                                           | 28   |
| 2. Separation of Methylated Nucleosides by Thin-Layer Chromatography . . . . .                                                         | 30   |
| 3. Separation of Ribosemethylated Nucleosides and Bases Excreted from Cultured Fibroblasts by Cation-Exchange Chromatography . . . . . | 47   |
| 4. Base and Ribosemethylated Nucleoside Composition of Hamster Embryo Fibroblast RNA . . . . .                                         | 55   |
| 5. Methylated Nucleoside Composition of Fibroblast RNAs . .                                                                            | 56   |
| 6. Effect of $\alpha$ -amanitin on Total RNA Levels . . . . .                                                                          | 58   |
| 7. Effect of $\alpha$ -amanitin on [ $^3\text{H-CH}_3$ ]-Methionine Labeling of RNA in Growing Fibroblasts. . . . .                    | 60   |
| 8. Effect of $\alpha$ -amanitin on RNA Specific Activity . . . . .                                                                     | 67   |
| 9. Effect of $\alpha$ -amanitin on the Excretion of Modified Nucleosides by Stimulated Fibroblasts . . . . .                           | 71   |
| 10. Effect of Actinomycin D on [ $^3\text{H-CH}_3$ ]-methionine Labeling of RNA in Serum Stimulated Fibroblasts . . . .                | 75   |
| 11. Effect of Antinomycin D on Modified Nucleoside Excretion by Serum Stimulated Fibroblasts . . . . .                                 | 76   |
| 12. Effect of Acetonitrile on Adsorption of Bases on Silica . . . . .                                                                  | 88   |
| 13. Effect of Acetonitrile on Adsorption of Nucleosides on Silica . . . . .                                                            | 89   |
| 14. Effect of Acetonitrile on Adsorption of Nucleotides on Silica . . . . .                                                            | 90   |
| 15. Effect of Acetonitrile on Efficiency of Group Separations . . . . .                                                                | 94   |
| 16. Effect of Acetic Acid on Nucleoside and Nucleotide Separations . . . . .                                                           | 95   |

| TABLE                                                         | PAGE |
|---------------------------------------------------------------|------|
| 17. Quantitation of Silica Chromatography . . . . .           | 97   |
| 18. Retention Volume for Methylated Nucleosides on Silica . . | 97   |
| 19. Retention Volume on Nucleic Acid Components on Silica . . | 98   |



## LIST OF FIGURES

| FIGURE                                                                                                                                        | PAGE |
|-----------------------------------------------------------------------------------------------------------------------------------------------|------|
| 1. Structures of Some Modified Nucleosides in RNA . . . . .                                                                                   | 2    |
| 2. Identification of Excreted Methylated Nucleosides and<br>Time Course of their Accumulation, Experimental Pro-<br>cedures . . . . .         | 12   |
| 3. $\alpha$ -amanitin Inhibition of RNA Synthesis, Experimental<br>Procedures . . . . .                                                       | 13   |
| 4. Effect of Methionine on Hamster Embryo Fibroblast<br>Growth . . . . .                                                                      | 24   |
| 5. Base Methylated Nucleosides Excreted by Cultured Fibro-<br>blasts . . . . .                                                                | 26   |
| 6. Thin-layer Chromatography of Ribothymidine . . . . .                                                                                       | 31   |
| 7. Thin-layer Chromatography of Dimethylguanosine . . . . .                                                                                   | 33   |
| 8. Thin-layer Chromatography of N-3-MethylCyd<br>N-1-Methyl . . . . .                                                                         | 36   |
| 9. Thin-layer Chromatography of N-1-MethylIno<br>N-1-MethylGuo . . . . .                                                                      | 38   |
| 10. Thin-layer Chromatography of N-5-MethylCyd<br>N-2-MethylGuo . . . . .                                                                     | 40   |
| 11. Thin-layer Chromatography of N-7-MethylGuo . . . . .                                                                                      | 42   |
| 12. Ribose Methylated Nucleosides and Methylated Bases<br>Excreted by Cultured Fibroblasts . . . . .                                          | 45   |
| 13. Excretion Kinetics of Base Methylated Nucleosides in Serum<br>Stimulated Fibroblasts . . . . .                                            | 51   |
| 14. The Effect of $\alpha$ -amanitin on [ $^3\text{H-CH}_3$ ]-Methionine Labeling<br>of the tRNA and rRNA in Stimulated Fibroblasts . . . . . | 61   |
| 15. Effect of $\alpha$ -amanitin on the Turnover of RNA in Stimulated<br>Fibroblasts . . . . .                                                | 64   |
| 16. Effect of Ammonium Borate on Nucleoside Retention on<br>Silica Cartridges . . . . .                                                       | 92   |

| FIGURE                                                                                                                                        | PAGE |
|-----------------------------------------------------------------------------------------------------------------------------------------------|------|
| 17. Salvage Pathways for Conversion of Ribothymidine<br>from RNA to Deoxythymidylate . . . . .                                                | 103  |
| 18. [ $^3\text{H-CH}_3$ ]-Methionine Labeled Ribonucleosides in Acid<br>Extracts of Cultured Fibroblasts . . . . .                            | 106  |
| 19. [ $^3\text{H-CH}_3$ ]-Methionine Labeled Ribose Methylated and<br>Deoxynucleosides in Acid Extracts of Cultured<br>Fibroblasts . . . . .  | 108  |
| 20. [ $^3\text{H-CH}_3$ ]-Methionine Labeled Ribonucleosides from<br>Alkaline Phosphatase Digestion of Acid-Soluble<br>Nucleotides . . . . .  | 110  |
| 21. [ $^3\text{H-CH}_3$ ]-Methionine Labeled Deoxynucleosides from<br>Alkaline Phosphatase Digestion of Acid-Soluble<br>Nucleotides . . . . . | 112  |

## CHAPTER I

### INTRODUCTION

Ribonucleic acids are degraded at different rates depending on the class of RNA and the physiological state of the cell (1-13). The mechanism of formation and the nature of intermediates of catabolism are not clear. Presumably, RNA is initially degraded to nucleotide fragments that are further degraded to nucleosides and nucleoside-phosphates which can be salvaged for reuse. It is not established if 3'-nucleoside-phosphates or 5'-nucleoside-phosphates are formed in the catabolism or both. However, only the 5'-nucleoside-phosphates of the major bases (Ade, Cyt, Ura, Gua, Thy) have previously been isolated from cells (14-15). Isolation of the 5'-phosphate nucleosides is to be expected since this is the precursor for growing polynucleotide chains in RNA and DNA synthesis.

Each of the several classes of RNA is characterized by a unique sequence derived from the DNA template and by the presence of a wide variety of base and sugar modifications. These modifications, of the four major bases, occur postranscriptionally at the polynucleotide level (16-24). Examples of a number of the minor bases which have been identified are illustrated in Figure 1. The vast majority of these compounds are synthesized in one step group transfer reactions (e.g., methylation, thiolation, acylation). Isomerization ( $\psi$ rd synthesis) and reduction (hU formation) are two additional types of enzymatic modification that have been demonstrated. The chemical structures of several

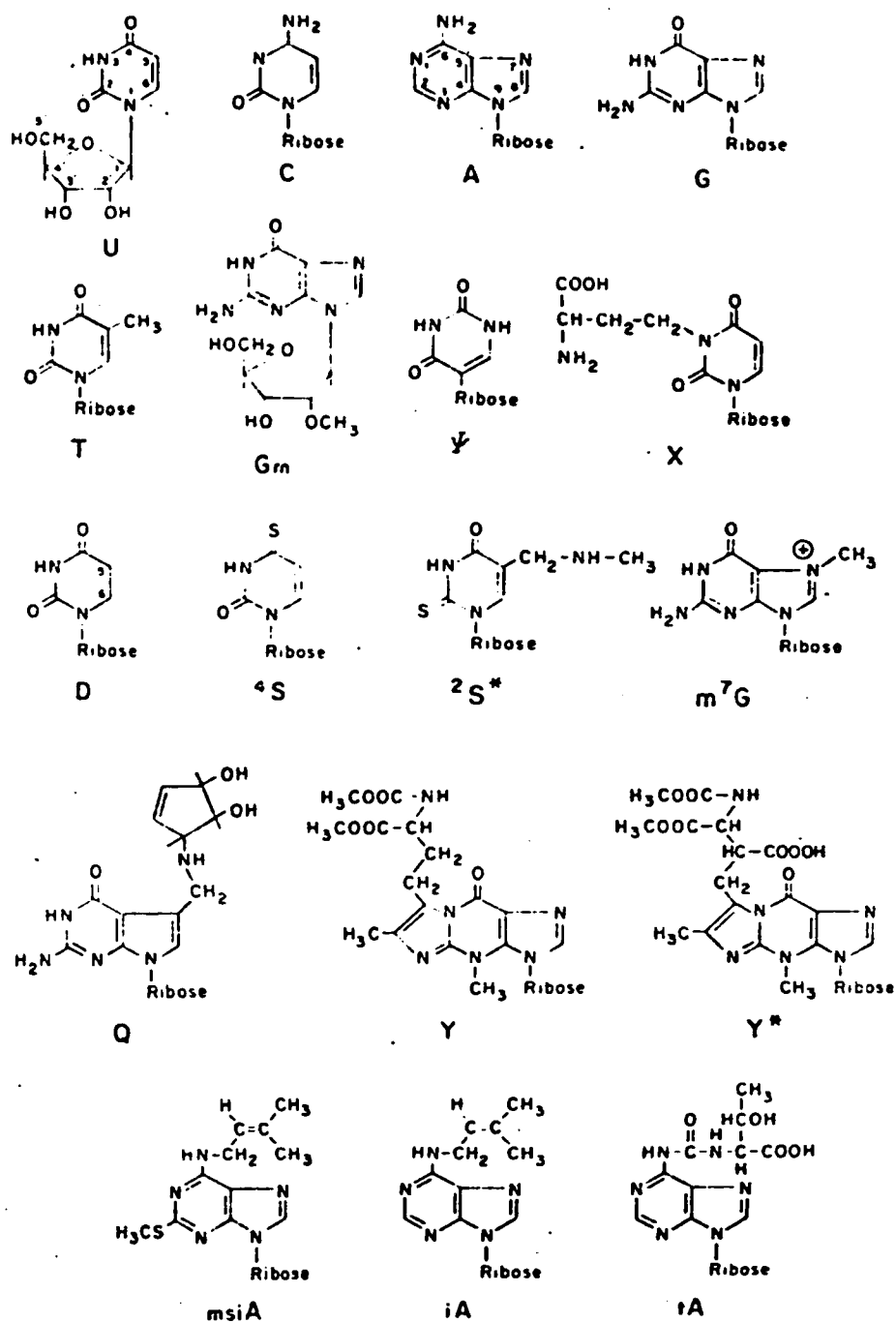


Figure 1. Structures of some modified nucleosides in RNA.

nucleosides (Q, Y, W) have also been identified. A nucleoside is considered to be hypermodified if its synthesis involves a series of successive reactions, rather than one step.

The diversity of known base modifications, their ubiquitous presence in RNAs and the appearance of specifically modified bases in characteristic positions, suggests they have an underlying role that relates to the overall function of RNA. An in-depth discussion of the biological roles of the modified nucleosides in RNA function is beyond the scope of this treatise, and would be a voluminous review in its own right. A number of excellent reviews on this subject have been published (25-33).

Since the modified bases, ubiquitous in all RNAs, are formed at the polynucleotide level, salvage and intracellular accumulation would be futile. Intracellular accumulation would, in fact, be disastrous to the cell. Incorporation of 2'-O-methyl nucleotides into growing RNA chains inhibits elongation and thus would block RNA synthesis (34). In addition, modified nucleosides that are incorporated in nucleic acids affect the fidelity of base pairing in transcription and translation (35). Mispairing would lead to errors in DNA replication as well as transcription and translation. Obviously, this would be catastrophic to the cell due to mutation induction and the synthesis of faulty enzymes. In addition, N-7-methylGuo and N-2-methylGuo nucleotides are known to inhibit protein synthesis (36-38).

Normally, the respective RNA and DNA polymerases do not insert these compounds into the growing chains. This is, presumably, because the modified nucleosides are not present as 5'-phosphates. Intracellular accumulation and incorporation into RNA of modified nucleosides could

occur if either 5' or 3'-phosphates were stable. In the absence of further catabolism or salvage, excretion of nucleosides is to be expected. In the intact animal, modified nucleosides and bases, intermediates of de novo purine and pyrimidine synthesis, and the major nucleosides in trace amounts are excreted in the urine (39).

A number of studies have been shown that there is increased excretion of modified nucleosides in tumor bearing animals as well as cancer patients (40-42). Increased excretion also has been correlated with other proliferative diseases including Hodgkin's disease, leukemia, and in an experimental granuloma in the rat (43-45). The basis for increased excretion is not understood. Generally, it has been felt that the appearance of modified nucleosides in urine was due primarily to tRNA turnover (40-47). This was because of the level of modification of tRNA and its constant turnover. Since it was felt excreted modified nucleosides were derived solely from tRNA turnover, it has been assumed that increased excretion is a reflection of changes in tRNA metabolism.

In agreement with this hypothesis, increased tRNA methylase activity in tumor cells has been well documented (48). However, additional experiments indicated the increased methylase activity is not always reflected in the tRNA base composition (49-56), but probably is a result of the absence of inhibitors of the methylases in tumor cells (48, 57-60). In addition, there are no general conclusions that can be drawn from the modified nucleoside content (increased vs. decreased) of tumor specific tRNAs except to say that the composition depends on the tRNA and the tissue examined (55-56).

It is not known if changes in the levels of the rRNA modification

enzymes occur in tumor cells, nor is there any data comparing the modified base compositions of rRNAs in normal and cancer cells. Also, there is no information comparing mRNA or nuclear RNA modification in normal and cancer cells. In short, there are no reports in the literature comparing the modification of RNAs other than tRNA in normal and cancer cells. Without that information, it would be difficult to unequivocally prove increased modified nucleoside excretion is a result of alterations in tRNA metabolism. Moreover, excreted nucleosides may be derived from rRNA as well as tRNA (this paper). If this were so, studies would necessarily have to be done comparing the modifications of all RNAs, not just tRNA, to determine the source of increased nucleoside excretion in cancer cells. The aim of this dissertation is to determine if tRNA is the sole source of excreted methylated nucleosides or if rRNA or other RNAs are sources as well.

There are several reasons to believe that rRNA should be a source of excreted modified nucleosides as well as tRNA. First, methylated nucleoside composition studies of eukaryotic RNAs indicate rRNA to be extensively modified (61-65), albeit not as much as tRNA (66-70). Secondly, rRNA is quantitatively the most important RNA in the cell with tRNA being the second most prevalent RNA. Finally, both classes of RNA are not stable, but turn over, although with different lifetimes. Upon catabolism, the modified nucleosides from both classes of RNA would be excreted. Since all RNAs turnover and RNA modification is ubiquitous, excreted nucleosides should be derived from all classes of RNA. In practicality, it would be difficult to demonstrate experimentally that mRNAs and nuclear RNAs contribute to excreted nucleosides without unique modifications of these RNAs since they

comprise about 5% of total RNA. However, if rRNA is a source of excreted nucleosides, this should be demonstrable.

The biochemical mechanisms regulating the excretion of modified nucleosides in intact animals are poorly understood and obscured by the physiology of blood transport, renal control and cell death. Thus, I have chosen to study the excretion of methylated nucleosides in cultured fibroblast cells as a model system to avoid the complications of nucleoside transport to the urine and cell death. The appearance of these nucleosides in the culture media is thus limited to their production by the cells.

Two groups of nucleosides can be produced; those derived from the purine and pyrimidine biosynthetic pathways (major nucleosides) and those produced by turnover of post-transcriptional modifications of RNA (minor nucleosides). Excretion of nucleic acid components from the former group has been observed (71-76). However, excretion of methylated nucleosides by cultured cells has not been rigorously demonstrated, although preliminary experiments indicate that minor nucleosides are excreted by cultured cells (71).

Excretion is to be expected if there is no intracellular accumulation via phosphorylation. Thus, the first objective of this dissertation is to determine if methylated nucleosides are excreted by cultured cells and to identify those nucleosides which are excreted.

Assuming that methylated nucleosides would be excreted by cultured cells, then my main objective is to use the cultured cell system to determine if rRNA is a source of excreted methylated nucleosides. That is, to determine if there is more than one RNA population which, upon turnover, contributes to the excreted nucleosides. If an



experimental condition could be devised so that only one of these RNA populations was being synthesized (i.e., labeled with [ $^3\text{H-CH}_3$ ]-methionine), then the excreted nucleosides (radioactivity in them) would reflect only that population, if indeed, both populations contribute to the excreted nucleosides.

In eukaryotic cells, there are three RNA polymerases which can be separated by their chromatographic properties. Each polymerase has a unique function. RNA polymerase I is located in the nucleolus and synthesizes rRNA (77-78). RNA polymerases II and III are located in the nucleoplasm and synthesize HnRNA, mRNA (77-79) and tRNA, 5sRNA (80-82) respectively. In addition to their specialized functions, the RNA polymerases are distinguished in vitro by their differential sensitivity to  $\alpha$ -amanitin, the sulphur containing bicyclic peptide from the mushroom "Amanita Phalloides" (80-83). Inhibition of Class II polymerases by very low levels of  $\alpha$ -amanitin (50% inhibition at 0.01 - 0.05  $\mu\text{g/ml}$ ) sets them apart from the other polymerases. Class III polymerases are at least 100 times less sensitive while Class I polymerases are resistant even to high levels (1000  $\mu\text{g/ml}$  for 50% inhibition) of the compound.

However, in vivo, RNA polymerase I as well as polymerase II is inhibited by low levels of  $\alpha$ -amanitin (84-88). The impairment of rRNA synthesis after blocking DNA-like RNA synthesis in vivo suggests that continuous synthesis of DNA-like RNA is necessary for the unimpaired functioning of the ribosomal RNA producing apparatus.

Also, when cultured mammalian cells are exposed to a relatively low concentration of actinomycin D, the synthesis of the 45S precursor of 18-28S rRNA is drastically inhibited, whereas there appears to be

little or no effect on the synthesis of tRNA, 5s rRNA and HnRNA (80-90).

By inhibiting the synthesis of rRNA but not tRNA, and comparing the radioactivity in the excreted methylated nucleosides in control and treated cells, I can determine if rRNA and tRNA are major sources of excreted nucleosides or only one or the other.

In summary, the major objectives of this dissertation are:

1. to determine if and which methylated nucleosides are excreted by cultured cells, and
2. to determine if rRNA and tRNA are both sources of excreted nucleosides or only one or the other.

## CHAPTER II

### EXPERIMENTAL PROCEDURES

#### Materials

Nucleosides, bases, and nucleotides were purchased from Cal Biochem., La Jolla, CA; Sigma, St. Louis, MO; and P-L Biochemicals, Milwaukee, WI. Acetonitrile ( $\text{CH}_3\text{CN}$ ) was obtained from Fischer Scientific Co., Fair Lawn, ND. (Inhalation and adsorption of acetonitrile should be prevented for it is a potent neurotoxin.) Commercially prepared silica cartridges (#51900) were purchased from Waters Associates, Milford, MA. Cation (A6) and anion-exchange (A28) resins were purchased from Bio-Rad Laboratories, Richmond, CA. Buffers were prepared from reagent grade chemicals or better from P-L, Sigma, or Mallinckrodt Chemical Works, St. Louis, MO.

Actinomycin D was purchased from Sigma,  $\alpha$ -amanitin from Cal Biochem., and [ $^3\text{H}$ - $\text{CH}_3$ ]-methionine (200mCi/mmol and 15 Ci/mmol) from New England Nuclear Corporation, Boston, MA. An additional mg of  $\alpha$ -amanitin, also from Cal Biochem, was the gift of S. K. Niyogi. Tissue culture media (H-21HG), special formula media (methionine free) and supplements (fetal calf serum, glutamine, and antibiotics) were obtained from Grand Island Biological Co., Grand Island, NY. Golden Syrian hamsters (10-12 days gestation) were purchased from Charles River Breeding Laboratory, Wilmington, MA. Stock solutions of methionine were prepared from crystalline methionine from Cal Biochem and sterilized by millipore (0.45 $\mu\text{m}$ ) filtration.

Spectrophotometric measurements were made with a Beckman DU

spectrophotometer. In all experiments radioactive samples were counted in a Beckman LS250 scintillation counter in a toluene based counting fluid.

### Tissue Culture

Hamster embryo fibroblasts (HEF) were prepared from pregnant hamsters (10-12 gestation) by dissecting the embryos, mincing the tissues, digesting with trypsin and plating the cells in 100 mm dishes. All experiments were done with cells on the third passage in subculture. Cells were normally cultured in Dulbecco's media (DMEM) supplemented with 10% fetal calf serum. Cells were grown in 8.5% CO<sub>2</sub> at 37°C and 98% relative humidity. Methionine free media was supplemented with sterile methionine as specified for cell growth in lower than optimal (30µg/ml in DMEM) methionine concentrations. Cells were routinely plated at  $2 \times 10^6$  cells/100 mm dish. Duplicate plates at each point were used for cell counts and for radioactive labeling. Cell counts were made with a Model A Coulter Counter or a hemocytometer.

### Experiment I - Identification of Excreted Nucleosides and Time Course of Their Accumulation in the Media

Cells were grown in media which contained 30µg/ml methionine for three days. At this time, the media was aspirated and cells refed fresh media which contained 1.5µg/ml methionine for two and one-half days. The media was aspirated and 20µCi of [<sup>3</sup>H-CH<sub>3</sub>]-methionine (200 mCi/mmole) was added in 10 ml fresh media (1.5µg/ml methionine). Cells were labeled for 23, 45, 60 and 81-1/2 hours. At these times the media was aspirated and frozen for later analysis. For identification of ribose methylated nucleosides cells were grown as described above and then labeled continuously with 30µCi of [<sup>3</sup>H-CH<sub>3</sub>]-methionine (15

Ci/mmol) for 8 1/2 hours. At this time, the media was aspirated and frozen for later analysis. A diagrammatic summary of these procedures is illustrated in Figure 2.

---

#### Experiment II - $\alpha$ -amanitin Inhibition of RNA Synthesis

Cells were grown in low methionine media as described above. Confluent cells were stimulated to grow by refeeding with fresh media five and one-half days after plating.  $\alpha$ -amanitin was added to the media at 1  $\mu$ g/ml, 60  $\mu$ g/ml or not at all at this time. After three hours, 30  $\mu$ Ci of [ $^3$ H-CH $_3$ ]-methionine (15.0 Ci/mmol) was added to control and treated cells for four hours. At this time, the media was aspirated, cells washed twice with 5 ml media (30  $\mu$ g/ml methionine), and finally with 10 ml fresh media (30  $\mu$ g/ml methionine) and allowed to grow in this media. Twenty-seven and 57 hours after the label was removed, the media was aspirated and frozen for later analysis from cells which received no  $\alpha$ -amanitin or had been treated with 1  $\mu$ g/ml or 60  $\mu$ g/ml  $\alpha$ -amanitin. The nucleic acids (RNA, DNA) were prepared for Sepharose 4B chromatography from these same cells as described below. A diagrammatic summary of these procedures is illustrated in Figure 3.

In an additional experiment, RNA synthesis was inhibited by treating cells with 6  $\mu$ g/ml  $\alpha$ -amanitin for three hours (point C, Figure 3), cells were then labeled (point D, Figure 3) with 45  $\mu$ Ci [ $^3$ H-CH $_3$ ]-methionine (15 Ci/mmol) for 5 hours. Cells which had been treated with  $\alpha$ -amanitin and cells with no treatment were washed as described above (point E) and the nucleic acids prepared after 40, 53, and 86 hours growth).

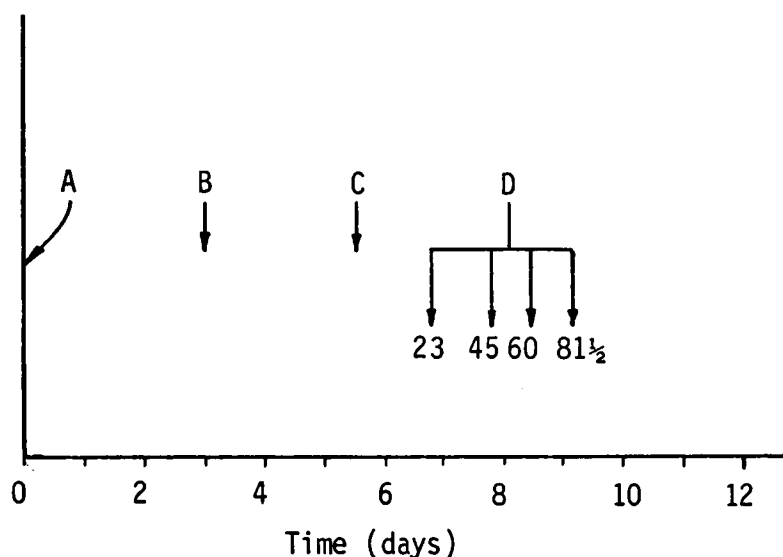


Figure 2. Identification of excreted methylated nucleosides and time-course of their accumulation, experimental procedures.

- (A) Cells were plated at  $2 \times 10^6$  cells/100mm dish ( $30\mu\text{g/ml}$ ). The numbers in parenthesis refer to the methionine concentration in the media.
- (B) The media was aspirated and the cells fed 10ml of fresh media ( $1.5\mu\text{g/ml}$ ).
- (C) The media was again aspirated and the cells fed 10ml fresh media ( $1.5\mu\text{g/ml}$ ) containing  $20\mu\text{Ci}$  [ $^3\text{H-CH}_3$ ]-methionine ( $200\text{mCi/mmole}$ ) or  $30\mu\text{Ci}$  [ $^3\text{H-CH}_3$ ]-methionine ( $15\text{ Ci/mmole}$ ) as specified.
- (D) Twenty-three, 45, 60 and/or  $81\frac{1}{2}$  hours after point C the media, from duplicate disks, were aspirated and frozen for later analysis.

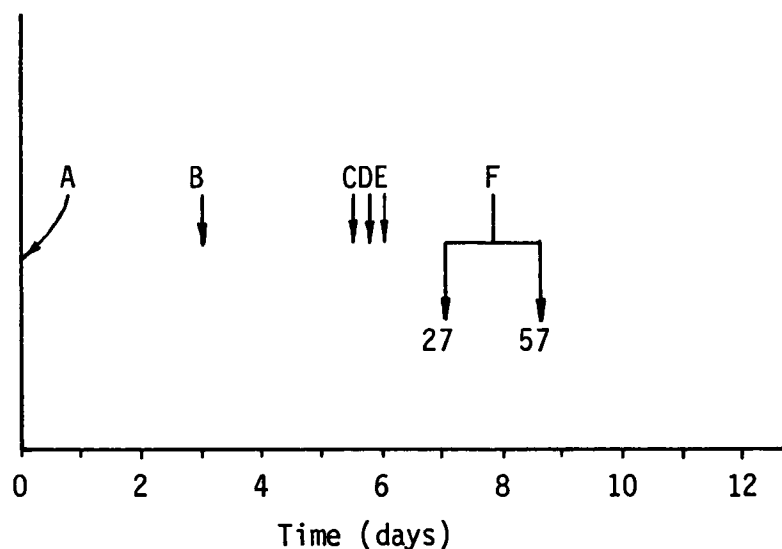


Figure 3.  $\alpha$ -amanitin inhibition of RNA synthesis, experimental procedures.

- (A) See legend to Figure 2.
- (B) See legend to Figure 2.
- (C) The media was aspirated and cells fed fresh media ( $1.5\mu\text{g/ml}$ ) supplemented with  $1\mu\text{g/ml}$   $\alpha$ -amanitin,  $60\mu\text{g/ml}$   $\alpha$ -amanitin or no  $\alpha$ -amanitin at all.
- (D) Three hours after point (C)  $30\mu\text{Ci}$  of  $[^3\text{H-CH}_3]$ -methionine ( $15\text{ Ci/mmol}$ ) was added to control and treated cells without changing the media.
- (E) Four hours after point (D) the media, containing the radioactive label and inhibitors, was aspirated; cells were washed twice with  $5\text{ ml}$  media ( $30\mu\text{g/ml}$ ) and finally with  $10\text{ ml}$  fresh media ( $30\mu\text{g/ml}$ ) and allowed to grow in this media.
- (F) Twenty-seven and 57 hours after point (E) the medias were aspirated and frozen for later analysis. The nucleic acids from the same cells were prepared for sepharose 4B chromatography.

### Experiment III - Actinomycin-D Inhibition of RNA Synthesis

Cells were grown in low methionine media as described in experiments I and II. Confluent cells were stimulated to grow with fresh media (low methionine) that had no actinomycin-D or had actinomycin-D added to the final concentration of  $0.04\mu\text{g/ml}$ . After two hours,  $30\mu\text{Ci}$  of [ $^3\text{H-CH}_3$ ]-methionine ( $15\text{ Ci/mmole}$ ) was added to each dish for two hours. At this time, the inhibitor and label were washed out, as in experiments I and II, and the cells allowed to continue to grow. Forty and 86 hours after removing the label, the media was aspirated and frozen and the nucleic acids prepared.

### Preparation of Nucleic Acids from Cultured Fibroblasts

After removal of media cells were scraped, collected in sterile tubes, centrifuged, and the cell pellets resuspended in  $0.1\text{ mg/ml}$  proteinase K in  $1.5\%$  SDS at pH 7.2 (91-94). The suspensions were incubated for six to eight hours at  $37^\circ\text{C}$ , and then frozen until further use. For cell number determinations, cells from a representative culture were removed from the culture dish by trypsinization and the cells counted with a hemocytometer.

Nucleic acids were isolated from the proteinase K digests after phenol extraction, since an SDS contaminant was found to cochromatograph in the tRNA region (95). The cell digest was extracted with 2 volumes of re-distilled, water-saturated phenol, and the aqueous layer reserved. The organic layer was re-extracted with an equal volume of water, and the aqueous portion combined with the first. The entire extraction procedure was repeated, and to the final aqueous layer was added 2-1/2 volumes of cold ethanol. RNA and DNA were



allowed to precipitate overnight.

The ethanol mixture, including the nucleic acid precipitate, was filtered through a millipore filter (0.45 $\mu$ m). The precipitate which collected on the filter was washed extensively with 70% ethanol. Material on the dried filter was dissolved in distilled water (1 ml) by soaking for a minimum of three hours. The solution was made of 20% N, N-dimethylformamide and applied to a column (0.9 x 100 cm) of 4B cross-linked Sepharose (sigma) equilibrated with the buffer: 0.1 M NaCl, 0.002 M HCOOH, 0.4 M NH<sub>4</sub>COOH, 0.02% NaN<sub>3</sub>, 20% DMF (95). Fractionation according to molecular weight was carried out at 48°C with the same buffer, and one ml fractions were collected. Absorbance at 260 and 280 nm was measured by a Beckman DB spectrophotometer. Column calibration was done with DNA and RNA standards.

From comparison of column profiles of proteinase K digests applied directly to the column with profiles of extracted digests, it was determined that the isolation procedure was quantitative and that no breakdown of the RNA had occurred (95). However, it should be emphasized that it is essential that only sterile disposable containers or proteinase K-incubated glass containers be used throughout this procedure. This precaution is necessary to prevent the breakdown of nucleic acids by ubiquitous nucleases.

#### Preparation of Acid-Soluble Pools from Cultured Cells and Media

Acid-soluble pools were prepared by treating cells with 5 ml ice-cold 0.5 M trichloroacetic acid for ten minutes at 3°C (96). Cells and supernatant were scraped from the dishes and each dish was washed with an additional 2 ml of the acid. This solution was

centrifuged for ten minutes at 16,000 x g to remove acid insoluble material. The supernatant fluid was neutralized and the acid extracted with 0.1 - 0.3 moles excess of a water-insoluble tertiary amine (Alamine 336, General Mills Chemicals, Kankakee, ILL. 55435) in a water-immiscible solvent (Freon TF, DuPont Co., Wilmington, DE 19898) as described by Khym (97). The aqueous phase was concentrated by evaporation (Rotary Evapo-Mix, Buchler Instruments, Fort Lee, NJ 07024). Acid-soluble pools were then processed on the silica cartridges and the nucleotide fractions concentrated and chromatographed on the anion exchange resin. (See Appendix I and reference 117 for the specifics.)

Acid-soluble pools from the media were extracted by adding 3 ml of 2 M TCA to 9 ml media for 20 minutes at room temperature to remove protein contaminants. The procedure was the same as for the pools from the cultured cells from this point.

#### Isolation of Nucleosides from Media

To isolate only nucleosides from the media, the following procedure was used. Nucleosides in 3 ml media were isolated by affinity chromatography on a borate gel, a cis-diol specific binder, as previously described (98). Bound nucleosides, which eluted in 31 ml of 1 M acetic acid, were concentrated by lyophilization and dissolved in 200  $\mu$ l of water.

#### Fractionation of Acid-Soluble Pools by Silica Chromatography

Apparatus for silica chromatography. The commercially packed silica cartridges (Water Associates) were inserted in the end of a 20

ml syringe (Becton-Dickerson and Co., Rutherford, NJ 08080). Buffers and samples were applied to the gels with a pasteur pipette and a flow rate of about 4 ml/min was maintained. After solutions were passed through the gel, the cartridge was removed from the end of the syringe and the plunger was withdrawn to refill the syringe.

Buffer preparations. We prepared solvents (v/v) by mixing acetonitrile with aqueous solutions of ammonium borate or acetic acid. For a solution of 90% acetonitrile, 10 ml water was mixed with 90 ml acetonitrile. Reagent-grade acetonitrile was filtered (Millipore 0.45- $\mu$ m membrane) to remove contaminants. Solutions were adjusted to 4 mM ammonium borate by addition of 200 $\mu$ l of 2 M ammonium borate (pH 7.8) per 100 ml of solution. Solutions were made 0.05 M in acetic acid by addition of 500 $\mu$ l of 10 M acetic acid per 100 ml of solution.

Sample loading and buffer change techniques. The apparatus employed for fractionations consisted of a syringe with luer end fitting, syringe plunger, several beakers to collect fractions, and the silica column itself. The plunger was fitted into the syringe following loading of samples and buffers, and the solution was then pushed through the column with a slight but steady pressure on the plunger (4 ml/min). As the liquid approached the top of the bed, the column was removed from the syringe while the plunger was still inside the syringe barrel. After the plunger was removed, the column was reinserted and the cycle could be repeated.

Column Equilibration, elution, and storage procedures. Prior to fractionation of base, nucleoside, and/or nucleotide mixtures, the dry silica columns were washed with 5 ml of 4 mM ammonium borate in water, then washed with 5 ml of 4 mM ammonium borate in 90% acetonitrile (equilibration buffer) or other solvents as indicated. Samples were composed of one volume of sample material mixed with nine volumes of acetonitrile containing borate: these were applied in 3 ml total volume of the equilibration buffer. The columns were washed with an additional 7 ml of equilibration buffer unless otherwise indicated. Nucleosides were eluted in 16 ml of 0.05 M acetic acid in 90% acetonitrile in water (v/v). Nucleotides were quantitatively eluted with 9 ml of water. After use, the columns were washed with 5 ml of water, dried with a stream of air, and stored at room temperature. The cartridges were prepared for reuse by drying for one hour at 130°C.

#### Anion-Exchange Chromatography

The anion-exchange resin was prepared and used as suggested by Khym (15,97). However, chloroform was used as the bacteriostatic agent rather than sodium azide and the gradient delay was eight minutes rather than six. Samples in 100  $\mu$ l or less were applied to the resin with an in-line injection device (model #7120, Rheodyne Co., Berkeley, CA 94710). The column dimensions were 300 mm x 3.9 mm and a flow rate of 0.7 ml/min. was maintained. The effluent was monitored at 254 nm and 280 nm. Quantitations were made by converting peak areas into nanomoles of substance (99). Individual values for molar absorptives (254 nm) were those determined by Khym (97).

### Cation-Exchange Chromatography

The methylated nucleosides were separated by cation exchange chromatography on a Bio-Rad A-6 column (70 cm x .6 cm) as previously described (100-101).

Nucleosides isolated by affinity absorption or silica chromatography were applied with the in-line injection device to a column equilibrated in a 0.4 M  $\text{NH}_4$  formate, 0.04 formic acid; pH 4.65 buffer and eluted in this buffer for three and one-half or four hours at which time the buffer was changed to 0.4 M  $\text{NH}_4$  borate; pH 8.1 for 1.5 hour. A flow rate of 0.4 ml/min. was maintained. Two minute fractions were collected and 200  $\mu\text{l}$  aliquots of each fraction counted in 2 ml of counting fluid.

### Thin-Layer Chromatography

Each of the isolated radioactive peaks separated by cation exchange chromatography was rechromatographed on cellulose thin-layer plates with one or more of the following solvent systems after desalting by affinity absorption (98), silica or charcoal (102) chromatography.

(A) 75 ml n-butanol/37.5 ml isobutyric acid/2.5 ml conc.  $\text{NH}_4\text{OH}$ /25 ml water.

(B) 120 ml isopropanol/ 50 ml 5%  $\text{NH}_4\text{Ac}$ , pH 4.65

(C) 170 ml n-butanol/ 30 ml water/ 1.5 ml conc.  $\text{NH}_4\text{OH}$

(D) Two-dimensional chromatography--solvent B for two hours followed by solvent A for two hours.

Chromatograms were developed in Kodak thin-layer plates for two and one-half to three hours. Radioactive unknowns were identified

by co-chromatography with authentic standards in sufficient quantities (0.01 to 0.05 O.D.) to see under a UV light. The thin-layers were dried and the patterns of UV absorbance were traced onto translucent paper. Five mm serial sections were cut from each lane and suspended in 200  $\mu$ l water plus 3 ml counting fluid.

#### Charcoal Desalting of Nucleic Acid Components

Methylated bases and ribose methylated nucleosides which had been separated by cation-exchange chromatography were desalted according to the procedure of Bandy and Cohn (102). Approximately one-half ml of charcoal was washed sequentially with 10 ml 50% isopropanol, 10 ml eluting solution (see below), 10 ml water then 15 ml 0.4 M HCl. Samples were adjusted to 0.3 M HCl, applied to the charcoal column which was then washed with 10 ml 0.01 M HCl. Nucleic acid components were then eluted with 20 ml eluting solution. Eluting solution--2 parts water/ 1 part conc.  $\text{NH}_4\text{OH}$ / 2 parts EtOH.

#### Enzymatic Digestion of RNAs for Methylated Nucleoside Compositions

Ribosomal RNA and tRNA from the cultured fibroblasts were purified and separated by molecular sieve chromatography as described above (see Figure 14 for a typical chromatogram). The fractionated RNAs were precipitated with ethanol, recovered by ultracentrifugation and resuspended in water. The RNAs were then digested to nucleosides according to the method of Uziel and Gassen (103).

#### Alkaline Phosphatase Digestion of Nucleotides

Nucleotides were extracted from the cells and separated from contaminants by silica chromatography as described above. Purified

nucleotides were digested with alkaline phosphatase, 1 mg/ml final conc., for 30 minutes at 37°C according to the procedure of Uziel and Gassen (103). The resulting nucleosides were subsequently fractionated into three groups (2'-O-methy and deoxy nucleosides; ribonucleosides; and charged nucleosides) by silica chromatography for analysis on the cation-exchange resin.

## CHAPTER III

### RESULTS AND DISCUSSION

#### Optimum Condition for Radioactive Labeling and Identification of Methylated Components of RNA that are Excreted by Cultured Cells

The first objective of these studies was to determine if cultured cells excrete minor nucleosides into the culture media. This would be analogous to their elimination in the urine in the whole animal. Thus, the experimental conditions require not only that RNA be synthesized and modified, but also that it be catabolized (i.e., turnover). During the turnover then, minor components may be excreted into the media.

Radioactive methionine can be used as a precursor to S-adenosyl methionine, the general methyl donor for minor nucleosides in RNA. As such, radioactive methionine is a specific label for methylated nucleosides. In addition, serum stimulation of quiescent fibroblasts to grow results in a synchronous induction of tRNA (104) and rRNA (105-107) synthesis and modification (108-111). Thus, I have labeled serum stimulated fibroblasts with [ $^3\text{H-CH}_3$ ]-methionine to specifically tag the methylated components of RNA. Confluent or stationary cells turnover RNA more actively than growing cells (1-8). Hence, methionine labeling of serum stimulated confluent cells seemed to be the optimum condition to determine if these compounds were excreted by tissue culture cells.

Methionine is an essential amino acid and is included in all media formulations. Hence, the specific activity of the radioactive



methionine would be diluted by a factor equal to the amount present in the media, plus that from protein turnover and intracellular pools. To maintain the radioactive methionine at the highest specific activity possible without interfering with cell growth, I have determined the minimum amount required for normal cell growth. In these experiments (Figure 4), I have cultured the fibroblast cells in special formulation methionine free media supplemented with sterile methionine at various concentrations. It is evident that a concentration of  $6\mu\text{g/ml}$  is equivalent to  $30\mu\text{g/ml}$  (amount normally present in DMEM media) for optimal cell growth while  $0.15\mu\text{g/ml}$  did not allow cell growth. In other experiments, it was observed that  $1.5\mu\text{g/ml}$  methionine was sufficient for growth although cells became stationary at a slightly lower (25% lower) total cell density.

#### Identification of Methylated Nucleosides Excreted by Cultured Fibroblasts

Base methylated nucleosides. [ $^3\text{H-CH}_3$ ]-methionine labeled nucleic acid components excreted into the culture media were purified for analysis by affinity absorption on a borate gel and/or adsorption to silica (see Appendix I) after acid extraction as described in the experimental procedures.

A typical profile of base-methylated nucleosides (Nucleoside fractions from silica chromatography) excreted by the fibroblast cells is illustrated in Figure 5. The retention volumes for the radioactive peaks were calculated and are tabulated in Table 1, as are values for the nucleoside standards corresponding to the radioactive peaks. These experiments tentatively identified the radioactive peaks as N-5

Figure 4. Effect of methionine on hamster embryo fibroblast growth. Cells were seeded at  $2 \times 10^6$  cells/100 mm dish and grown in 10 ml methionine-free media supplemented with  $0.15 \mu\text{g/ml}$  ( $\Delta$ ),  $6 \mu\text{g/ml}$  ( $\bullet$ ), and  $30 \mu\text{g/ml}$  (O) methionine. At three days the media was changed in all cultures. Cell counts were made with a model A Coulter counter. Each point is an average of two independent experiments.

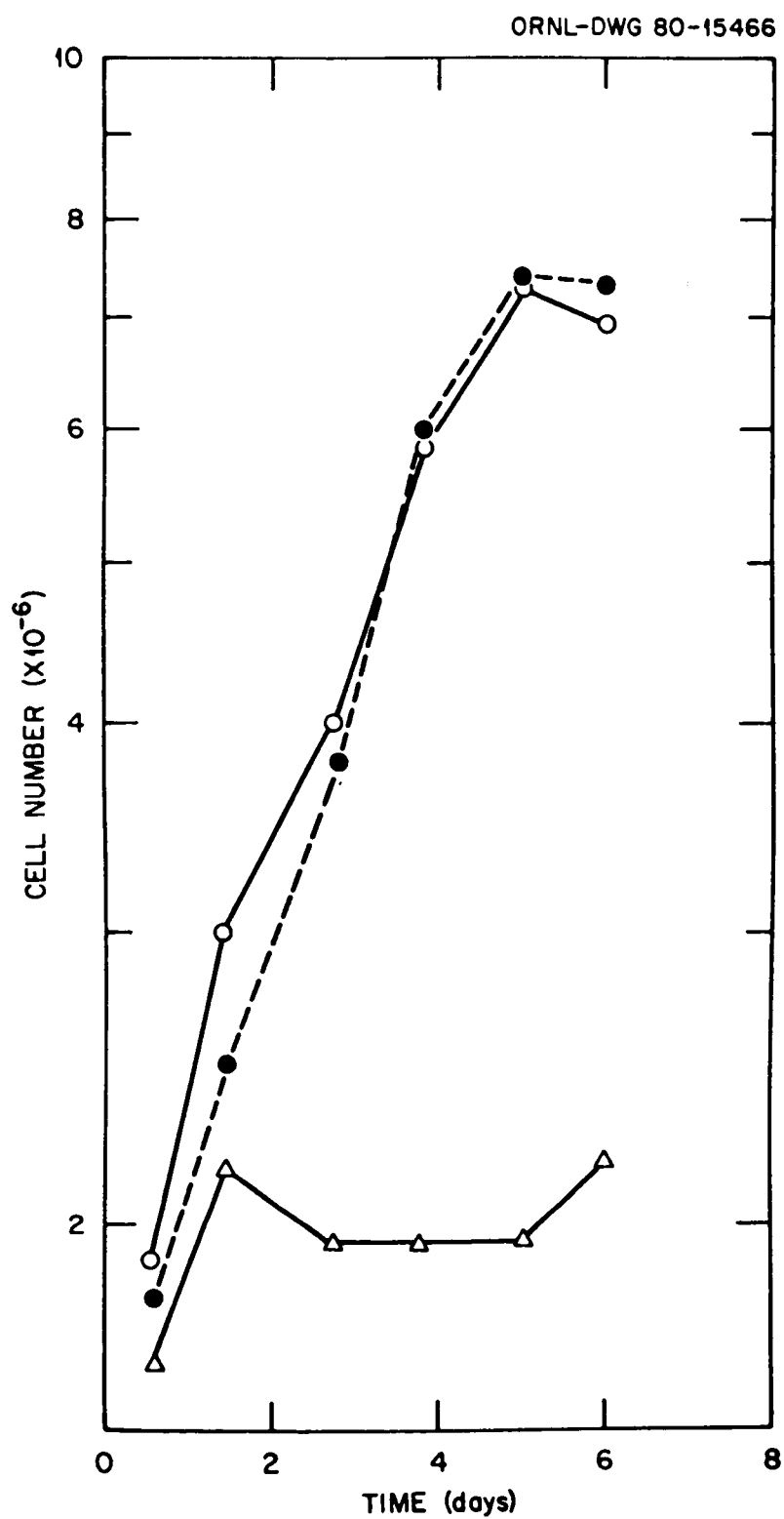


Figure 4

Figure 5. Base methylated nucleosides excreted by cultured fibroblasts. Fibroblasts were cultured, labeled with [ $^3\text{H}$ -CH $_3$ ]-methionine, and the nucleosides purified by affinity absorption on a borate gel. The acetic acid wash from the borate gel run was lyophilized and dissolved in water. An aliquot was chromatographed on the cation-exchange resin as described in the Experimental Procedures section, and fractions (0.8 ml) were collected every two minutes.

ORNL-DWG 80-15460

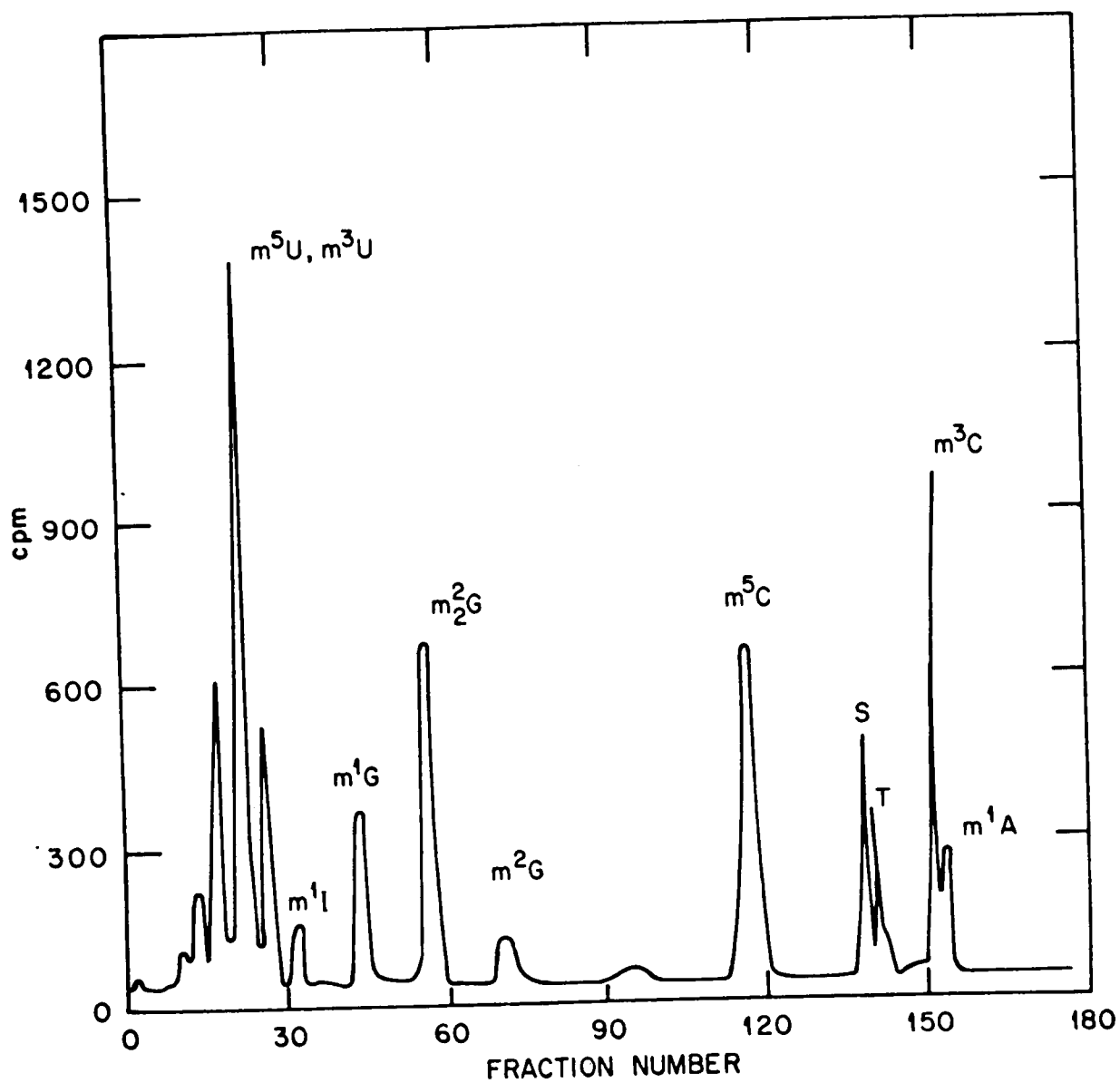


Figure 5

TABLE 1  
SEPARATION OF EXCRETED METHYLATED NUCLEOSIDES BY  
CATION-EXCHANGE CHROMATOGRAPHY<sup>1</sup>

| Nucleosides                   | Retention Volumes (ml) |                                                                   |
|-------------------------------|------------------------|-------------------------------------------------------------------|
|                               | Nucleoside Standards   | [ <sup>3</sup> H-CH <sub>3</sub> ]-Methionine Labeled Nucleosides |
| m <sup>5</sup> U              | 18                     | 18                                                                |
| m <sup>1</sup> I              | 26                     | 26                                                                |
| m <sup>1</sup> G              | 37                     | 35                                                                |
| m <sub>2</sub> <sup>2</sup> G | 45                     | 46                                                                |
| m <sup>2</sup> G              | 57                     | 57                                                                |
| m <sup>5</sup> C              | ND <sup>2</sup>        | 94                                                                |
| m <sub>2</sub> <sup>6</sup> A | 110                    | 111                                                               |
| m <sup>7</sup> G              | ND <sup>2</sup>        | 113                                                               |
| m <sup>1</sup> A              | 122                    | 122                                                               |
| m <sup>3</sup> C              | 124                    | 124                                                               |

<sup>1</sup>See the experimental procedures for column specifications and chromatographic conditions.

<sup>2</sup>ND = Not determined directly, the retention volumes were calculated (100 - 101).

methylUrd (ribothymidine); N-1 methylIno; N-1-MethylGuo; N-2-dimethylGuo, N-2-methylGuo; N-5-methylCyd; N-1-methylAdo, and N-3-methylCyd.

To establish the identity of the radioactive peaks separated on the cation-exchange resin, they were pooled, concentrated and chromatographed in one or several thin layer chromatography systems.  $R_F$  value for the nucleoside standards in the several solvent systems are listed in Table 2.

Thin-layer chromatography of the pooled radioactivity from the peak at 18 ml plus authentic ribothymidine showed a coincidence of UV and the radioactive counts in solvents A and C (Figure 6). In both solvent systems, an additional radioactive peak was observed which chromatographs as N-3 methylUrd. In solvent A, the radioactivity coincident with authentic ribothymidine was 51% of the recovered CPM and the remaining chromatographed as N-3-methylUrd. In solvent C 48% was found in N-5-methylUrd while the remaining CPM chromatographed as N-3-methylUrd. In a two-dimensional thin-layer system (solvent B for two hours followed by solvent A for two hours), 50% of the peak at 18 ml (A6 column) was coincident with ribothymidine. These results demonstrate the peak at 18 ml consists of two components, N-3-methylUrd and N-5-methylUrd.

To confirm that the radioactive peak at 46 ml (A6 column) was N-2-dimethylGuo, an aliquot was chromatographed in solvent A with authentic N-2-dimethylGuo. Two radioactive peaks were observed. One peak had an  $R_F$  characteristic N-2-dimethylGuo, and was coincident with the UV peak (Figure 7). Eighteen percent of the recovered counts was coincident with N-2-dimethylGuo, and the remainder migrating as the faster component. The more rapid moving band is the free base,

TABLE 2  
SEPARATION OF METHYLATED NUCLEOSIDES BY  
THIN-LAYER CHROMATOGRAPHY<sup>1</sup>

| Nucleoside Standards | $R_F$     |           |           |
|----------------------|-----------|-----------|-----------|
|                      | Solvent A | Solvent B | Solvent C |
| $m^5U$               | 53        | 72        | 41        |
| $m^1I$               | 48        | 65        |           |
| $m^1G$               | 46        | 61        |           |
| $m^2G$               | 48        | 63        |           |
| $m_2^2G$             | 53        | 65        |           |
| $m^5C$               | 52        | 61        |           |
| $m^7G$               | 54        | 51        |           |
| $m_2^6A$             | 93        |           |           |
| $m^6A$               |           | 75        |           |
| $m^3U$               | 43        | 86        | 83        |
| $m^1A$               | 53        | 53        |           |
| $m^3C$               | 47        | 61        |           |

<sup>1</sup>See Experimental Procedures for solvent compositions and chromatographic conditions.



Figure 6. Thin-layer chromatography of ribothymidine. Chromatography was as described in the Experimental Procedures section.

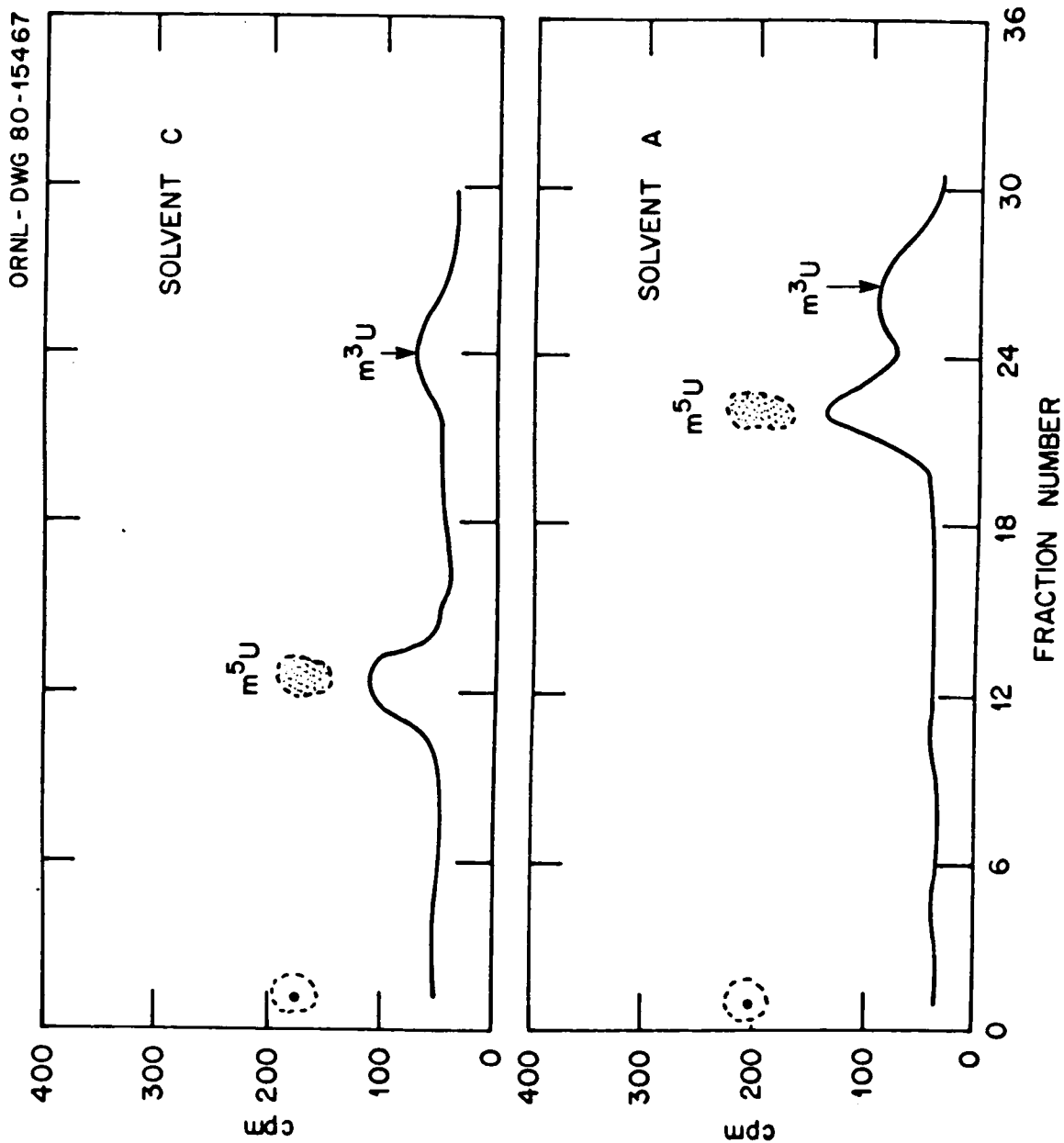
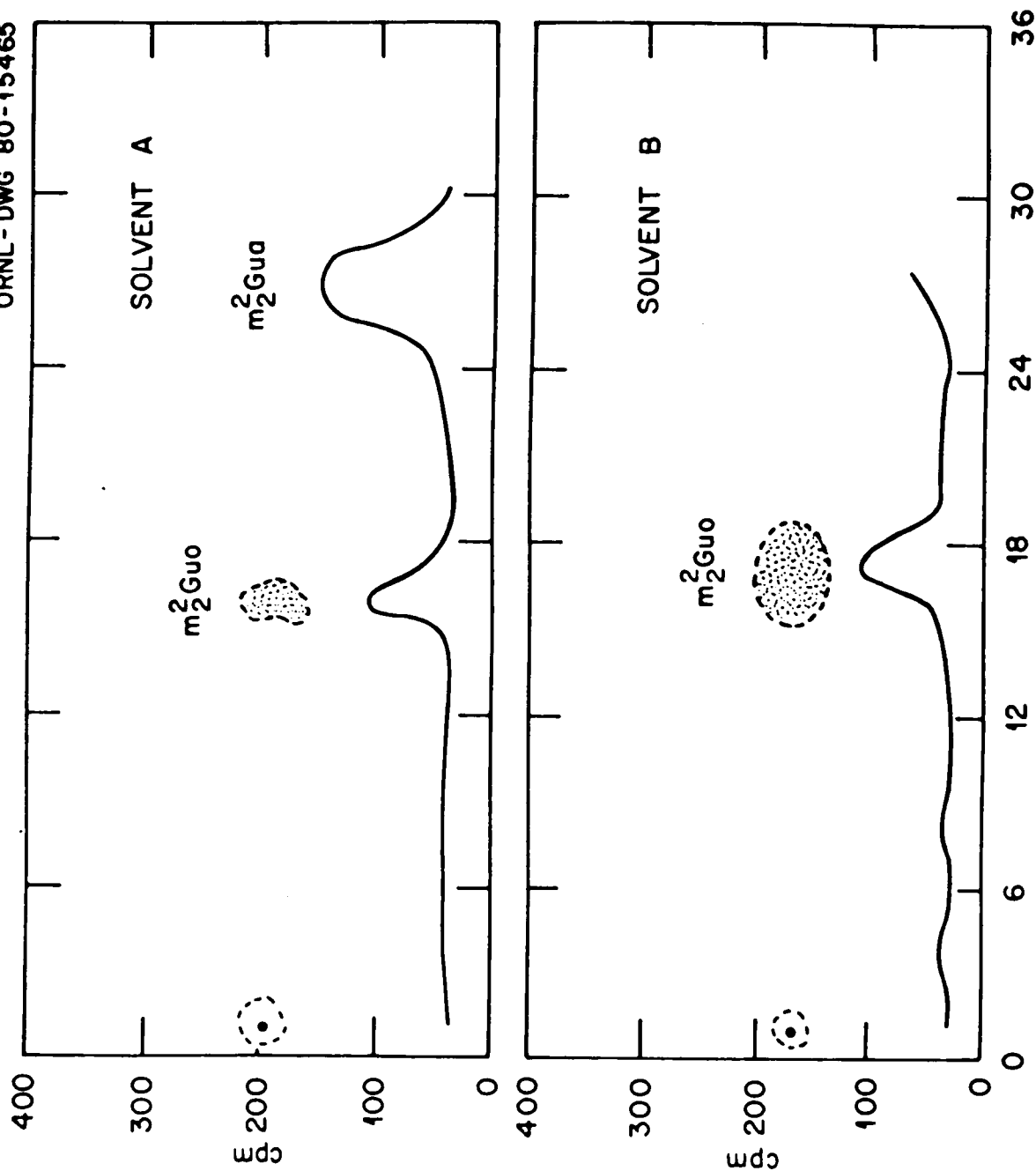


Figure 6

Figure 7. Thin-layers chromatography of dimethylguanosine. Chromatography was as described in the Experimental Procedures section.

ORNL - DWG 80-15465

FRACTION NUMBER  
Figure 7

N-2-dimethylGua. Partial acid hydrolysis of authentic N-2-dimethylGuo results in two UV bands in solvent A with the same  $R_F$  values as the original dimethylGuanosine and the free base (not shown).

Chromatography of an additional aliquot of the peak at 46 ml in the higher pH solvent (solvent B) resulted in two radioactive peaks with an increase in the proportion of N-2-dimethylGuo. The remaining radioactivity was present at the solvent front with N-2-dimethylGua. These results confirm the identification as N-2-dimethylGuo.

When authentic N-1-methylAdo was chromatographed in solvent B, an additional UV absorbing band was observed which had an  $R_F$  characteristic of N-6-methylAdo. At the pH of this solvent N-1-methylAdo can rearrange to N-6-methylAdo. Thus, when the radioactive peaks at 122 and 124 mls were run in the second dimension, three radioactive peaks were observed (Figure 8). One was coincident with authentic N-1-methylAdo, another with authentic N-3-methylCyd, and a third with authentic N-6-methylAdo. Seventy-one percent of the cpm applied on the thin layer plate were recovered. N-1-methylAdo was 64%, N-3-methylCyd was 20%, and N-6-methylAdo was 7% of the recovered counts, respectively.

Similar workup was employed for the radioactive peaks at 26, 35, 57, and 94 mls. The results (Figure 9, 10) indicated the peaks chromatographed as single components and were coincident with N-1-methylIno, N-1-methylGua, N-2-methylGua and N-5-methylCyd, respectively. These results support the identifications by cation-exchange chromatography.

When the putative N-7-methylGuo peak (T) at 113 ml was chromatographed with authentic N-7-methylGuo in solvent A (Figure 11), two radioactive peaks were observed. Neither peak was coincident with authentic N-7-methylGuo. Fifty-four percent of the recovered cpm was in peak 1

Figure 8. Thin-layer chromatography of N-3-MethylCyd and N-1-MethylAdo. Chromatography was as described in the Experimental Procedures section.

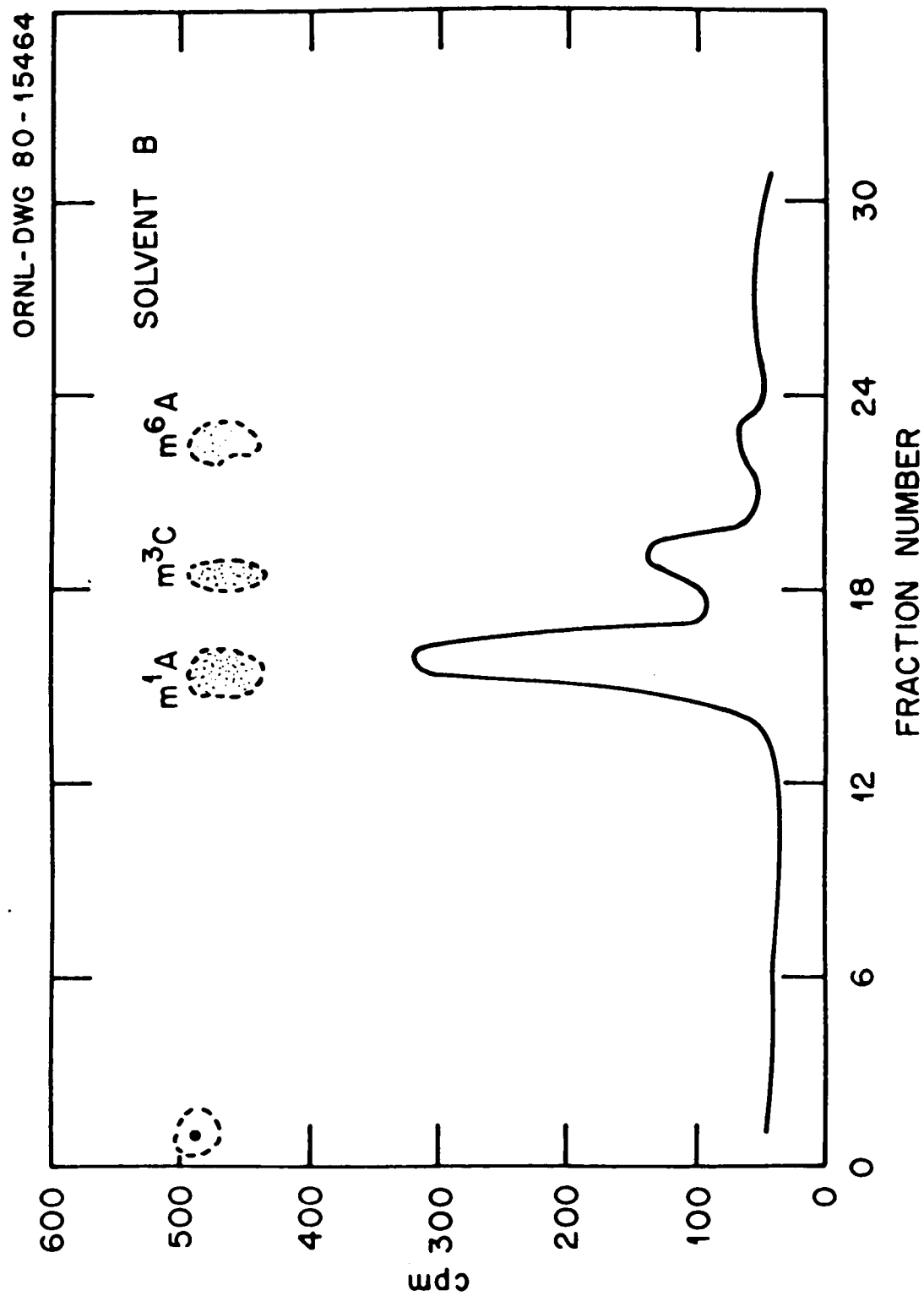


Figure 8

Figure 9. Thin-layer chromatography of N-1-methylIno and N-1-methylGuo. Chromatography was as described in the Experimental Procedures section.



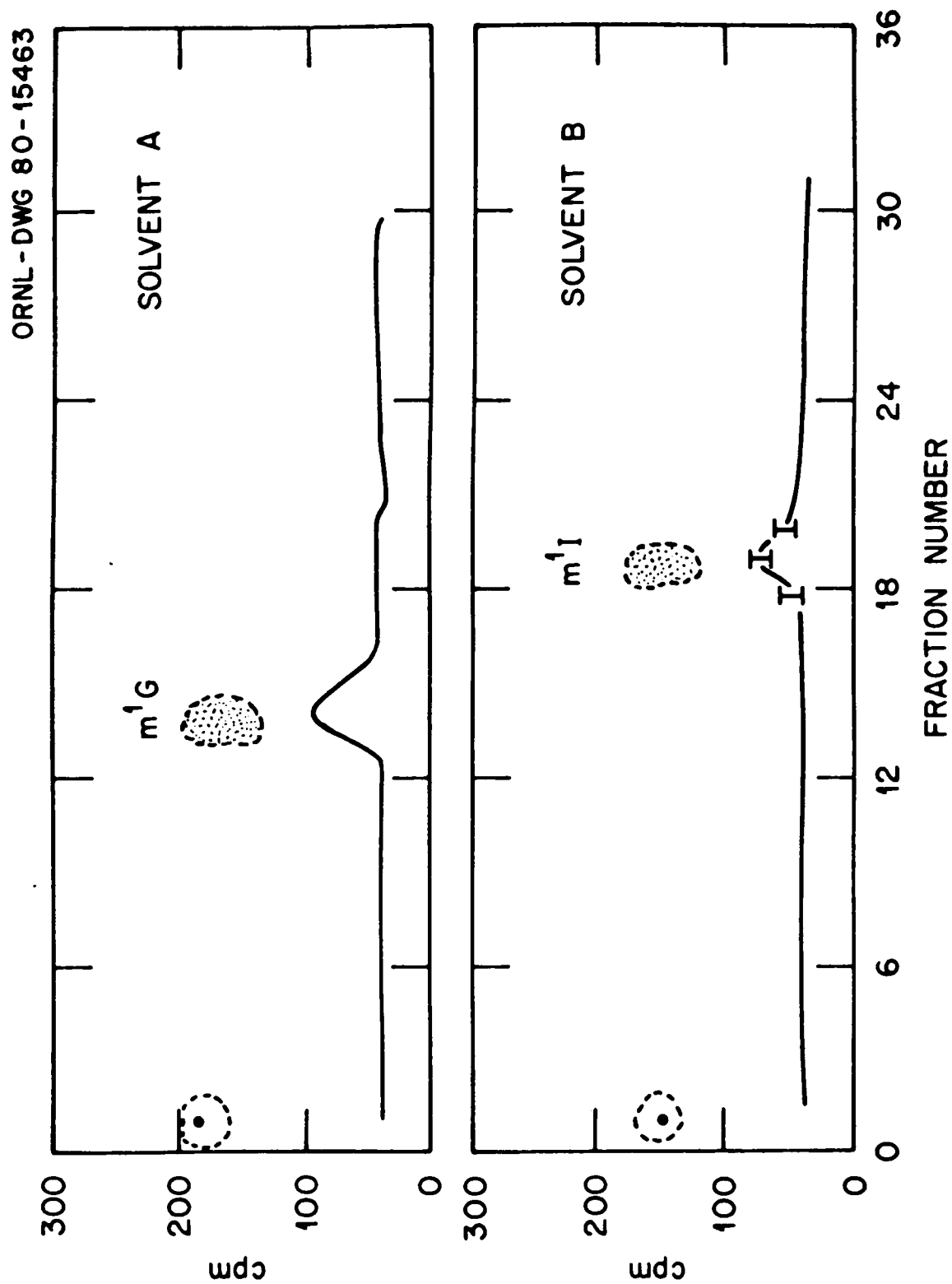


Figure 9

Figure 10. Thin-layer chromatography of N-5-methylCyd and N-2-methyl-Guo. Chromatography was as described in the Experimental Procedures section.

ORNL-DWG 80-15462

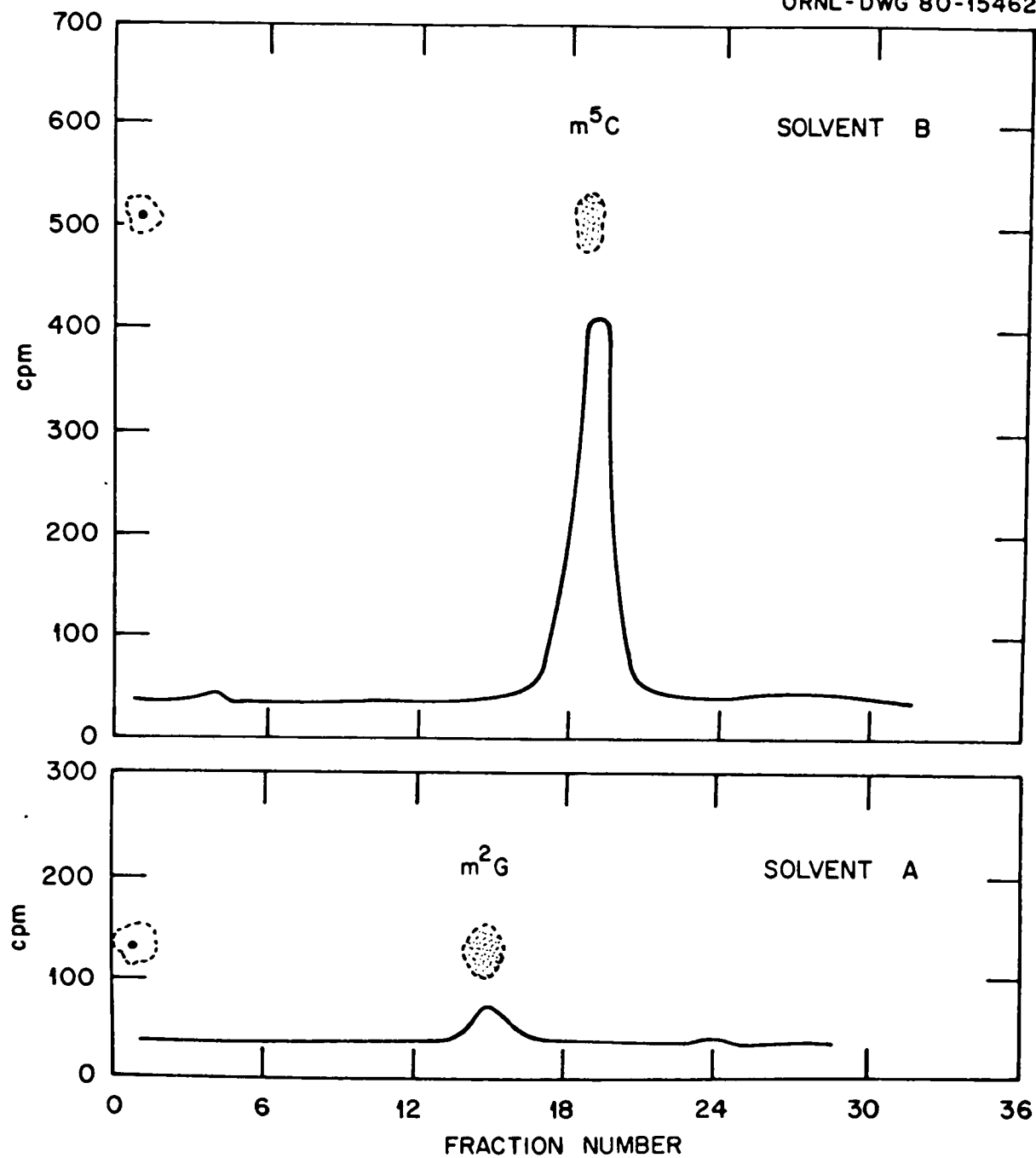


Figure 10

Figure 11. Thin-layer chromatography of N-7-methylGuo. Chromatography was as described in the Experimental Procedures section.

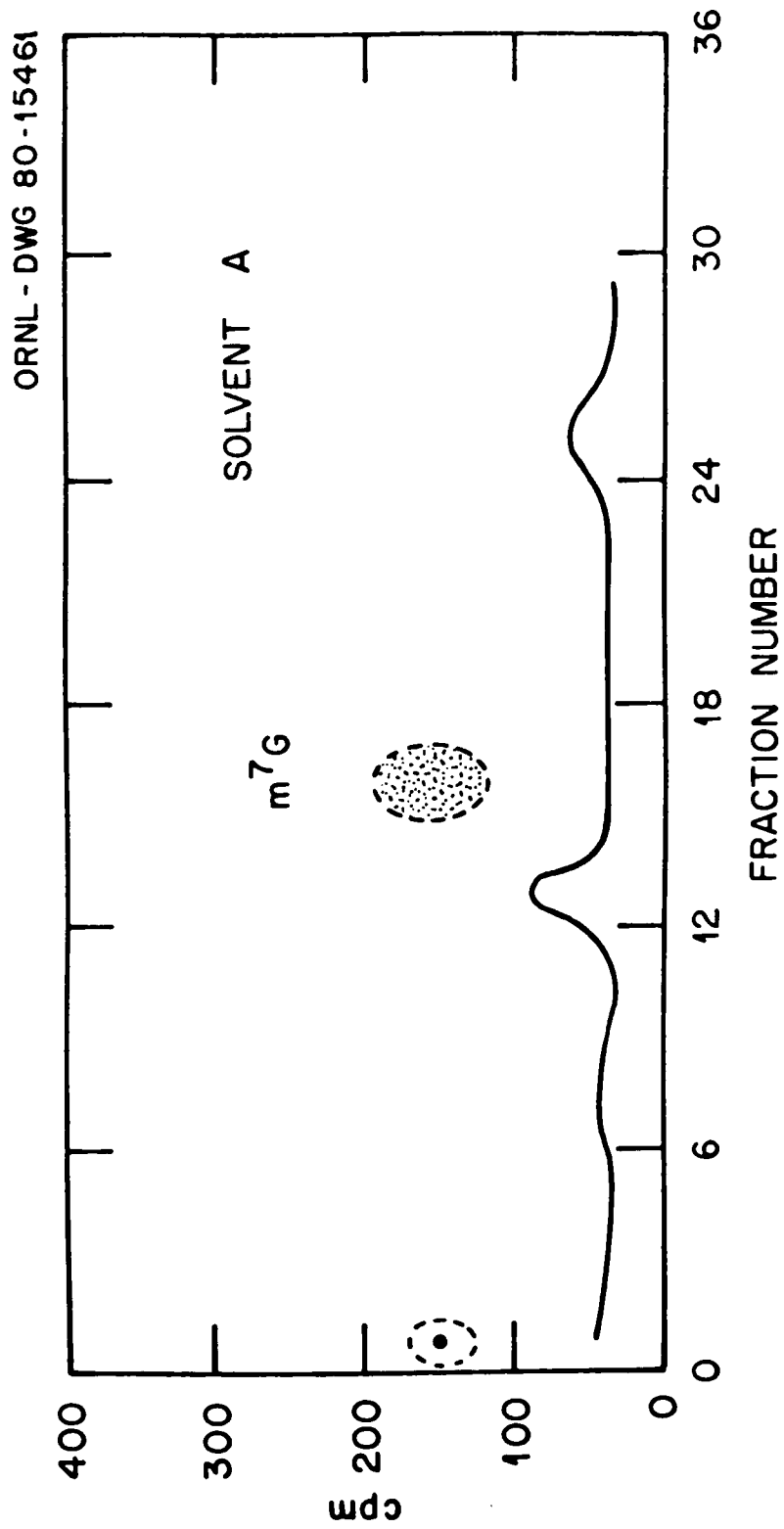


Figure 11

and 46% in peak 2. The radioactive peak at 113 ml has yet to be further characterized, although it does not appear to be N-7-methylGuo.

The putative N-6-dimethylAdo peak at 111 mls (S) was chromatographed in solvents A and C with authentic N-6-dimethylAdo. The results from these experiments did not confirm the peak at 111 mls as N-6-dimethylAdo (not shown). This peak also has not been further characterized.

Ribose methylated nucleosides and methylated bases. A typical profile of ribose methylated nucleosides and methylated bases excreted by the hamster embryo fibroblasts is illustrated in Figure 12. The retention volumes for the radioactive peaks and the nucleoside and base standards to which they correspond are listed in Table 3. These experiments suggested that ribose methylated nucleosides excreted by the fibroblast cells included 2'-O-methylUrd, 2'-O-methylGuo, 2'-O-methylIno and 2'-O-methylCyd while 2'-O-methylAdo was not a significant excretion product of the cells. Two additional radioactive peaks were tentatively identified as thymidine, and N-7 methyl Guanine.

To confirm the identity of the radioactive peaks separated on the cation-exchange resin, they were pooled, desalted on charcoal, concentrated and chromatographed in the two-dimensional thin layer system. These experiments confirmed the identification obtained by cation-exchange chromatography. For example, when the radioactive peaks at 52 ml (2'-O-MeGuo) and 84 ml (2'-O-MeCyd) were chromatographed in solvent B for two hours then solvent A for two hours with authentic standards, 86% and 99% respectively, of the applied radioactivity was coincident with the corresponding UV spot. Similar

Figure 12. Ribose methylated nucleosides and methylated bases excreted by cultured fibroblasts. Fibroblasts were cultured, labeled with [ $^3\text{H-CH}_3$ ]-methionine and the excreted nucleosides were prepared by silica chromatography, prior to cation-exchange chromatography, as described in the Experimental Procedures section.

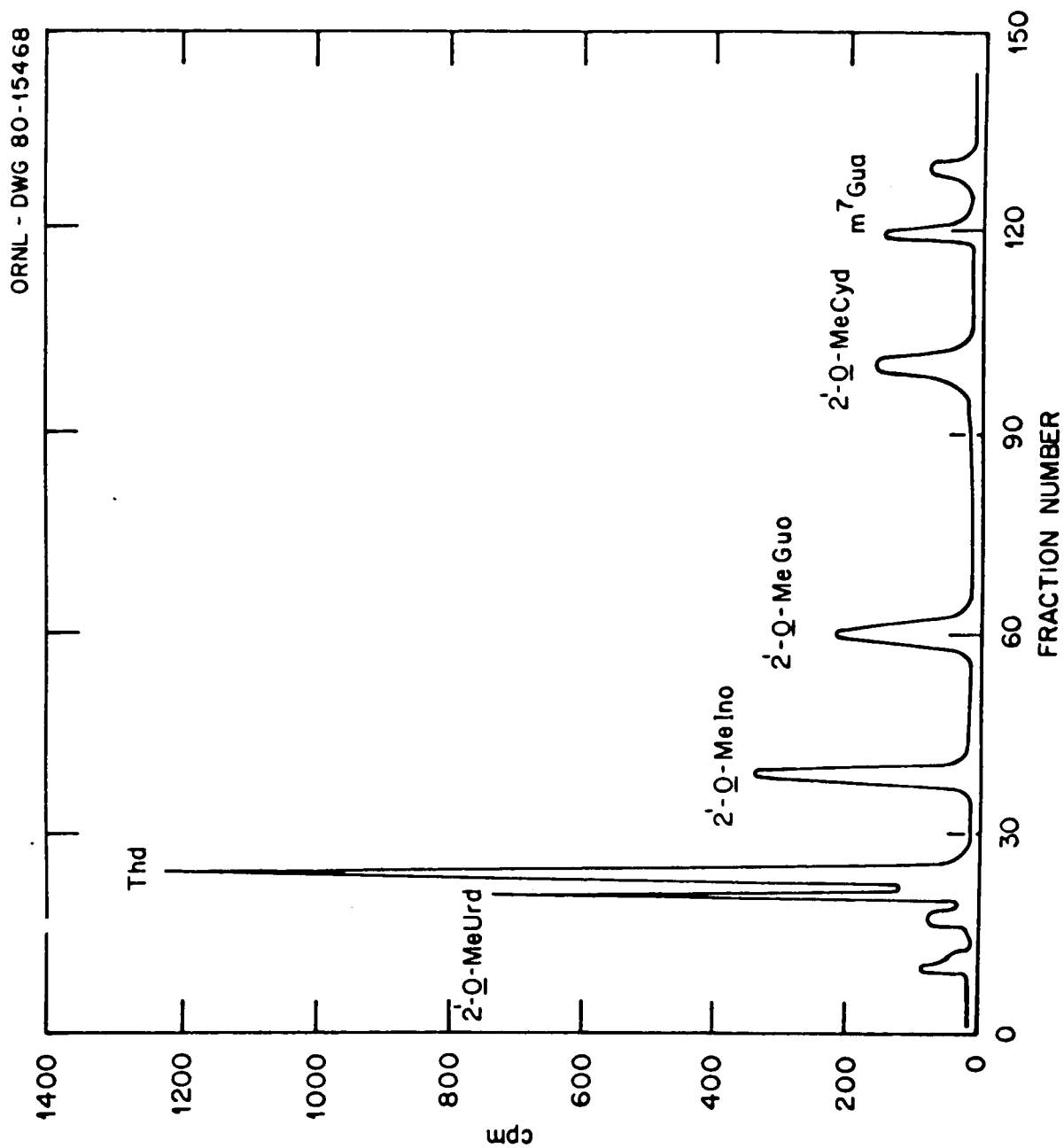


Figure 12



TABLE 3

SEPARATION OF RIBOSEMETHYLATED NUCLEOSIDES AND BASES EXCRETED FROM CULTURED FIBROBLASTS BY CATION-EXCHANGE CHROMATOGRAPHY<sup>1</sup>

| Compounds               | Retention Volume              |                                                                 |
|-------------------------|-------------------------------|-----------------------------------------------------------------|
|                         | Nucleoside and Base Standards | [ <sup>3</sup> H-CH <sub>3</sub> ]-Methionine Labeled Compounds |
| 2'- <u>O</u> -MethylUrd | 18                            | 18                                                              |
| Thymidine               | 20                            | 20                                                              |
| 2'- <u>O</u> -MethylIno | ND <sup>2</sup>               | 32                                                              |
| 2'- <u>O</u> -MethylGuo | 52                            | 51                                                              |
| 2'- <u>O</u> -MethylAdo | 76                            |                                                                 |
| 2'- <u>O</u> -MethylCyd | 84                            | 85                                                              |
| N-7-MethylGua           | 102                           | 101                                                             |

<sup>1</sup>See the Experimental Procedures section for column specifications and chromatographic conditions.

<sup>2</sup>ND = Not determined directly, the retention volume was calculated (100 - 101).

experiments were carried out with the other radioactive peaks and, in all cases, the results confirmed the identifications.

In summary, a number of methylated nucleosides ( $m^2_2G$ ,  $m^2G$ ,  $m^1G$ ,  $m^5U$ ,  $m^3U$ ,  $m^1A$ ,  $m^3C$ ,  $m^5C$ ,  $m^1I$ ,  $C_m$ ,  $U_m$ ,  $G_m$ ,  $I_m$ , Thd) and one methylated base ( $m^7Gua$ ) have been identified as excretion products of the cultured fibroblasts. In this respect then, a tissue culture system is similar to the whole animal in that the methylated nucleosides are excreted. In the intact animal (open system) these compounds are eliminated in urine while in a tissue culture system (closed system) they are accumulated in the media.

Several differences between the tissue culture system and the whole animal are worth noting. For instance, in human urine only the base, thymine is excreted, the nucleoside ribothymidine is not present in urine. In the tissue culture system, ribothymidine is a major excretion product. Secondly, in urine methylated bases as well as methylated nucleosides are observed but in the tissue culture system the methylated nucleosides are the final end product of catabolism. Only in the case of N-7-methylGua is the base excreted and for this particular compound the nucleoside was not observed. Apparently, with cultured cells there is a very specific nucleosidase for N-7-methylGuo which rapidly lyses the nucleoside to the free base (Uziel personal communication), but does not catabolize the other methylated nucleosides (Uziel, Keyes, and Cornelius unpublished).

It is anomalous that the [ $^3H-CH_3$ ] group of methionine labels thymidine since folate is the methyl donor rather than Adenomet in the de novo synthesis of thymidylate (112). On the other hand, Adenomet is the active methyl donor in ribothymine synthesis in RNA. This

observation suggests a salvage pathway for converting rThd to Thd is operating in these cells. A complete discussion and evidence supporting the proposed salvage pathway for rThd is described in Appendix II. Ribothymidine in this respect is different from the other methylated nucleosides that are excreted. It can be salvaged and converted to deoxythymidine for use in DNA synthesis as well as excreted. In fact, ribothymidine is the only methylated nucleoside that is isolatable as a nucleotide in the acid-soluble pools (see Appendix II). The other methylated nucleosides, end products of RNA metabolism are not salvaged and are of no known use to the cell.

In fact, accumulation of certain methylated bases ( $m^7G$ ,  $m^1G$ ) to a high level would be detrimental and transform normal cells to malignant ones (113). As demonstrated above, except for N-7-methylGuo, which is catabolized to the base N-7-methylGua the methylated nucleosides are not catabolized significantly past the nucleoside levels. Thus alterations in RNA metabolism would be reflected in the pattern of excretion of the methylated nucleosides and N-7-methylGua.

#### Excretion Kinetics of Methylated Nucleosides

The time course of accumulation of base methylated nucleosides in the media was determined by continuous labeling of fibroblasts with [ $^3H-CH_3$ ]-methionine and measuring the amounts of radioactivity in several of the methylated nucleosides at 23, 45, 60, and 81-1/2 hours after growth stimulation. The nucleosides were prepared and analyzed as described in the experimental procedures. Absolute quantitation of the nucleoside levels was not made since only the amount of radioactivity was determined not the specific activity. This was because the amounts

of these compounds in the media was below the detection limits of my analytical system. In addition, there are a number of other UV absorbing compounds in the media that interfere with analysis by adsorption alone even after partial fractionation (98). Measurements of radioactivity indicate that these nucleosides accumulate exponentially until a maximum level is reached at 60 hours following serum stimulation to grow and that level is maintained for at least 20 more hours (Figure 13A and 13B). This description of the excretion kinetics appears to be true for all the nucleosides measured except N-2-methylGuo which decreases slightly during the last 20 hours.

Quantitation of excreted pseudouridine, also a modified nucleoside, in these same fibroblasts shows a maximum rate of excretion in growing cells but as the cells became confluent the rate of excretion of  $\psi$ rd is reduced (71). This observation is analogous to my observations on excretion of methylated nucleosides by serum stimulated fibroblasts. When the cells are stimulated to divide by changing the media there is a burst of RNA synthesis, breakdown and excretion. As the cells enter quiescence, again, the excretion rate of the methylated nucleosides is diminished.

#### Methylated Base Composition of Ribosomal and Transfer RNA from Stimulated Fibroblasts

The methylated base compositions of rRNA and tRNA from hamster embryo cells were compared to compositions from other eukaryotic cells (61-64; 68-70; 114-115). Ribosomal and transfer RNA were labeled, purified and digested, as described in the Experimental Procedures.

Figure 13. Excretion kinetics of base methylated nucleosides in serum stimulated fibroblasts. Cells were cultured, labeled with [ $^3\text{H}$ -CH<sub>3</sub>]-methionine, and the nucleosides measured as described in the Experimental Procedures section.

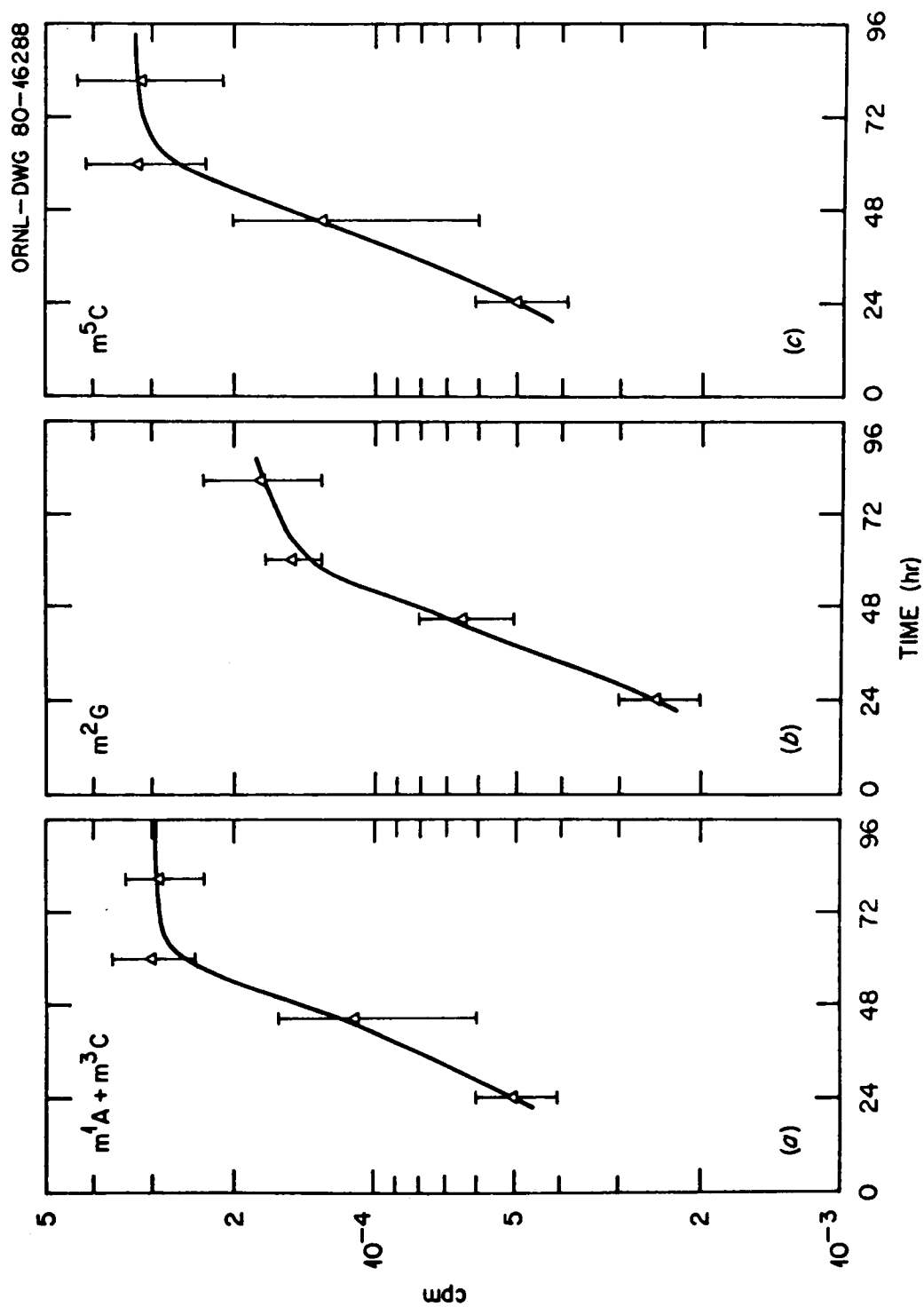


Figure 13

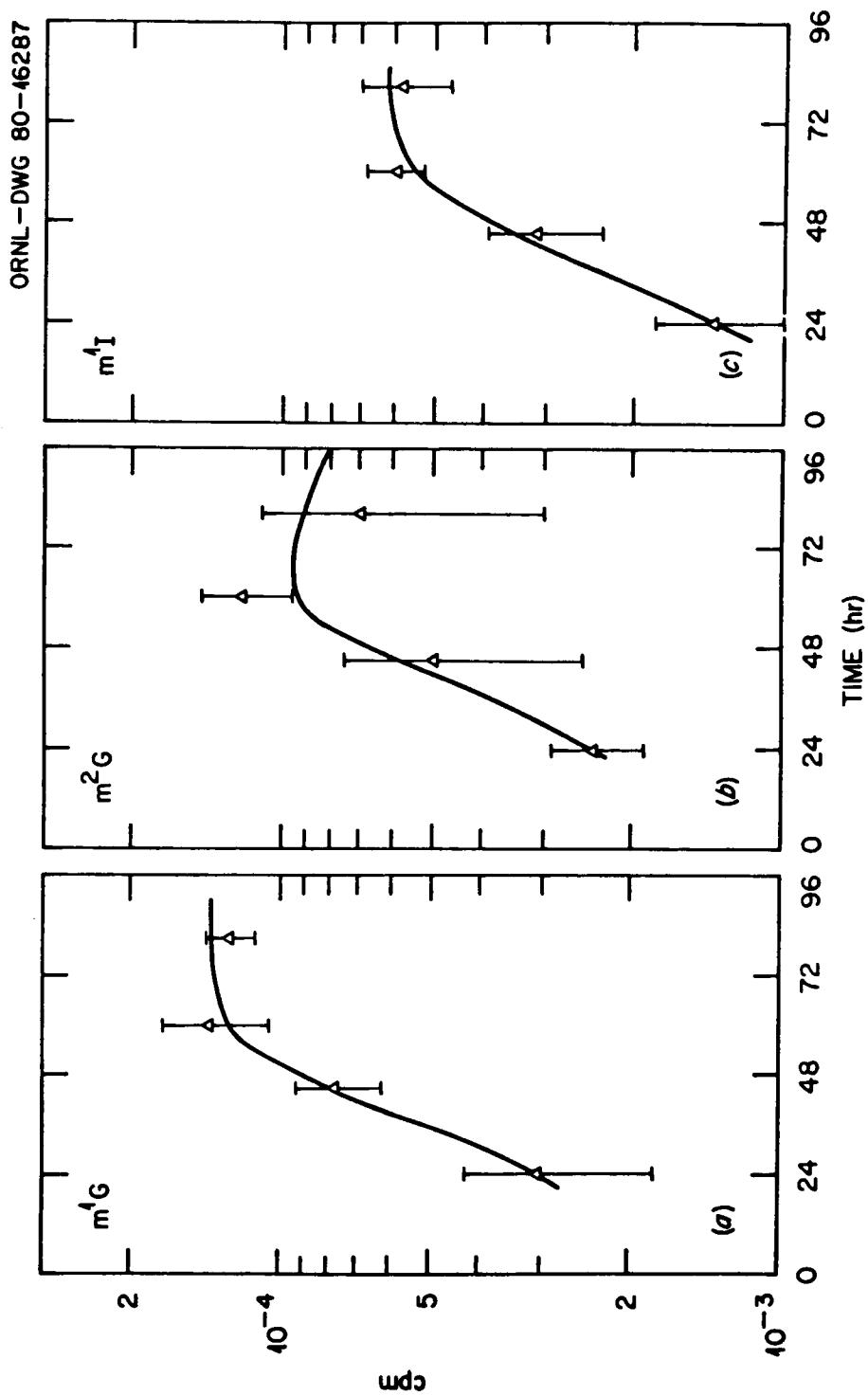


Figure 13 (Continued)

The nucleosides from the digested RNAs were separated into 2'-O-methyl nucleosides, base methylated nucleoside and charged nucleoside groups by silica chromatography (Table 4).

Analysis of the individual methylated nucleosides in tRNA and rRNA was made by chromatographing each group on the cation-exchange resin and determining the radioactivity in each compound (Table 5). The 2'-O-methyl nucleosides are the major methylated nucleosides in rRNA while the base methylated nucleosides are the major methylated components of tRNA. A significant amount of the 2'-O-methyl nucleosides are present in tRNA as well.

These observations (compositional data) are consistent with methylated nucleoside compositions determined independently by a number of other investigators (61-64; 68-70; 114-115).

It is interesting that 2'-O-methylAdo is a major 2'-O-methylated nucleoside in both tRNA and rRNA while 2'-O-methylIno is not detected. 2'-O-methylAdo is not recovered from the culture media but 2'-O-methylIno is a major excretion product of the fibroblast cells. This suggests that the 2'-O-methylAdo liberated from RNA breakdown is deaminated to form 2'-O-methylIno. In support of this Kelly and his colleagues (116) have noted the high level of Adenosine deaminase activity in cultured fibroblasts and in the culture media.

#### Effect of RNA Synthesis Inhibitors on RNA Metabolism and Nucleoside Excretion

Inhibition of rRNA synthesis by  $\alpha$ -amanitin. As discussed in the introduction it is my opinion that ribosomal as well as tRNA should be



TABLE 4  
BASE AND RIBOSE METHYLATED NUCLEOSIDE COMPOSITION  
OF HAMSTER EMBRYO FIBROBLAST RNA<sup>1</sup>

| RNA  | % of Total          |                   |
|------|---------------------|-------------------|
|      | Ribose Methylations | Base Methylations |
| tRNA | 27.7%               | 72.3%             |
| rRNA | 61.9%               | 38.1%             |

<sup>1</sup> RNAs were [<sup>3</sup>H-CH<sub>3</sub>]-methionine labeled, purified, separated, and digested to nucleosides as described in the experimental procedures. The nucleosides were then separated into base methylated and ribose-methylated groups by silica chromatography.

TABLE 5  
METHYLATED NUCLEOSIDE COMPOSITION OF FIBROBLAST RNAs<sup>1</sup>

| Nucleoside                        | tRNA | rRNA |
|-----------------------------------|------|------|
| A <sub>m</sub>                    | 3.6  | 14.2 |
| C <sub>m</sub>                    | 4.6  | 13.5 |
| U <sub>m</sub>                    | 5.6  | 12.4 |
| G <sub>m</sub>                    | 4.4  | 15.3 |
| N-1 <sub>m</sub>                  | 7.5  | 6.5  |
| N-2 <sub>m</sub>                  | 2.0  |      |
| m <sup>5</sup> U/m <sup>3</sup> U | 38.5 | 27.0 |
| m <sup>1</sup> I                  | 0.7  |      |
| m <sup>1</sup> G                  | 1.6  | 0.8  |
| m <sub>2</sub> <sup>2</sup> G     | 1.2  |      |
| m <sup>5</sup> C                  | 8.2  | 1.4  |
| m <sup>6</sup> A                  | 1.1  |      |
| m <sup>2</sup> G                  | 6.2  | 0.5  |
| m <sup>1</sup> A/m <sup>3</sup> C | 3.8  | 0.9  |
| mN-3                              | 11.1 | 7.5  |

<sup>1</sup> RNAs were prepared, digested, and separated into ribose methylated and base methylated groups by silica chromatography. Each group was then chromatographed on the cation-exchange resin to determine the individual methylated nucleosides in each group. N-1<sub>m</sub> and N-2<sub>m</sub> are unidentified ribose methylated nucleosides and mN-3 is an unidentified base methylated nucleoside. The values for the individual peaks are summarized as the percent radioactivity recovered.

a source of excreted modified nucleosides although there is no proof of this. An objective of this dissertation, then is to determine if there is more than one RNA population which upon turnover contributes to the excreted nucleosides. If an experimental condition could be devised in which only one of these RNA populations was being synthesized (i.e., labeled with [ $^3\text{H-CH}_3$ ]-methionine) then the levels of excreted nucleosides (radioactivity) would measure only that population.

The synthesis of eukaryotic rRNA can be selectively inhibited with  $\alpha$ -amanitin (84-88) and Actinomycin D (89-90). This can be used to determine if rRNA is a source of excreted nucleosides by momentarily inhibiting the synthesis of rRNA, but not tRNA, in the presence of a radioactive precursor for either class of RNA, then returning the cells to normal growth, and comparing the radioactivity in the excreted nucleosides in control and treated cells.

The effect of  $\alpha$ -amanitin on the synthesis, methylation and turnover of RNA in the stimulated fibroblasts was investigated to determine if any adverse effects of  $\alpha$ -amanitin on these processes would preclude this experimental design for investigating the origin of excreted modified nucleosides.

RNA synthesis was inhibited with  $\alpha$ -amanitin in cells stimulated to grow by addition of fresh serum. Cells were labeled with [ $^3\text{H-CH}_3$ ]-methionine and the RNA prepared as described in the Experimental Procedures.

The total amounts ( $A_{260}$ ) of ribosomal and tRNA were determined after purification and size fractionation on the Sepharose-4B column (Table 6). During the 60 hour recovery period the levels of rRNA increase in cells which received no  $\alpha$ -amanitin, remain about the same in

TABLE 6  
EFFECT OF  $\alpha$ -AMANITIN ON TOTAL RNA LEVELS<sup>1</sup>

| $\alpha$ -Amanitin | rRNA               | tRNA                | rRNA                | tRNA                 |
|--------------------|--------------------|---------------------|---------------------|----------------------|
|                    | $A_{260}$          |                     | $A_{260}$           |                      |
|                    | 27 hours           |                     | 60 hours            |                      |
| None               | 3.35 $\pm$<br>0.35 | 0.93 $\pm$<br>0.24  | 4.1 $\pm$<br>0.38   | 0.48 $\pm$<br>0.02   |
| 1 $\mu$ g/ml       | 3.35 $\pm$<br>0.21 | 0.615 $\pm$<br>0.05 | 3.25 $\pm$<br>0.27  | 0.36 $\pm$<br>0.13   |
| 60 g/ml            | 2.8 $\pm$<br>0.42  | 0.54 $\pm$<br>0.21  | 0.96 $\pm$<br>0.035 | 0.296 $\pm$<br>0.025 |

<sup>1</sup>The effect of  $\alpha$ -amanitin on total RNA levels was determined by measuring the total absorbance of a given peak 27 and 60 hours after treating the cells as described in the Experimental Procedures section. The data has been normalized to the dna content of the cultures ( $A_{260}^{\text{RNA}}/A_{260}^{\text{DNA}}$ ).

cells treated with  $1\mu\text{g/ml}$   $\alpha$ -amanitin and decrease significantly in cells treated with  $60\mu\text{g/ml}$   $\alpha$ -amanitin. It is noteworthy that rRNA quantities, at 27 hours post-treatment, are the same for the control and  $1\mu\text{g/ml}$  treated cells but considerably less in the cells treated with  $60\mu\text{g/ml}$   $\alpha$ -amanitin.

The quantities of tRNA are as expected considerably less than the levels of rRNA. The (tRNA) quantity decreases during the 60 hour recovery period in both the control and  $\alpha$ -amanitin treated cells. The absolute amount of tRNA, however, is inversely related to the  $\alpha$ -amanitin dose.

The effect of  $\alpha$ -amanitin on the methylation of RNA can be determined since the cells were labeled with [ $^3\text{H-CH}_3$ ]-methionine during the last three hours of the  $\alpha$ -amanitin treatment (Table 7). The extent of methylation was measured with the same cells that the RNA levels were determined. The most striking conclusion from these experiments is the apparently complete absence of labeling of rRNA size molecules in cells treated with  $60\mu\text{g/ml}$   $\alpha$ -amanitin (Figure 14). The extent of rRNA methylation is equivalent in control and  $1\mu\text{g/ml}$   $\alpha$ -amanitin treated cells. Methylation of tRNA is not inhibited by either  $1\mu\text{g/ml}$  or  $60\mu\text{g/ml}$   $\alpha$ -amanitin. In fact, as the  $\alpha$ -amanitin concentration is increased there is an increased incorporation of radioactive methionine into tRNA size molecules.

When the total methylation (tRNA + rRNA) is compared in control,  $1\mu\text{g/ml}$  and  $60\mu\text{g/ml}$  treated cells there is apparently no significant or only minor differences. The molecular events responsible for the apparent compensation in total methylation when rRNA methylation is inhibited are considered below.

TABLE 7  
EFFECT OF  $\alpha$ -AMANITIN ON [ $^3\text{H-CH}_3$ ]-METHIONINE LABELING  
OF RNA IN GROWING FIBROBLASTS<sup>1</sup>

| $\alpha$ -Amanitin  | tRNA                                                    |                   | rRNA                          |                   | Total              |                   |
|---------------------|---------------------------------------------------------|-------------------|-------------------------------|-------------------|--------------------|-------------------|
|                     | Hours after removal of [ $^3\text{H-CH}_3$ ]-methionine |                   |                               |                   |                    |                   |
|                     | 27                                                      | 60                | 27                            | 60                | 27                 | 60                |
| None                | 4554 $\pm$<br>1095                                      | 2255 $\pm$<br>714 | 2979 $\pm$<br>494             | 1968 $\pm$<br>238 | 7533 $\pm$<br>1589 | 4223 $\pm$<br>952 |
| 1 $\mu\text{g/ml}$  | 3727 $\pm$<br>419                                       | 2144 $\pm$<br>250 | 2891 $\pm$<br>24              | 1658 $\pm$<br>274 | 6618 $\pm$<br>443  | 3802 $\pm$<br>524 |
| 60 $\mu\text{g/ml}$ | 5958 $\pm$<br>1288                                      | 3357 $\pm$<br>760 | No significant<br>label found |                   | 5958 $\pm$<br>1288 | 3357 $\pm$<br>760 |

<sup>1</sup>The data has been normalized (CPM/A<sub>260</sub>DNA) to the DNA content of the cultures, as determined by the absorbance at 260 nm (Sepharose 4B column), and is an average of two determinations.

Figure 14. The effect of  $\alpha$ -amanitin on [ $^3\text{H-CH}_3$ ] methionine labeling of the tRNA and rRNA in stimulated fibroblasts. Cells were grown, labeled with [ $^3\text{H-CH}_3$ ]-methionine, and the nucleic acids prepared for Sepharose-4B chromatography as described in the Experimental Procedures section. Cells were treated with  $1\mu\text{g/ml}$  (o—o),  $60\mu\text{g/ml}$   $\alpha$ -amanitin (●—●) supplemented media or only media ( $\Delta$ — $\Delta$ ).

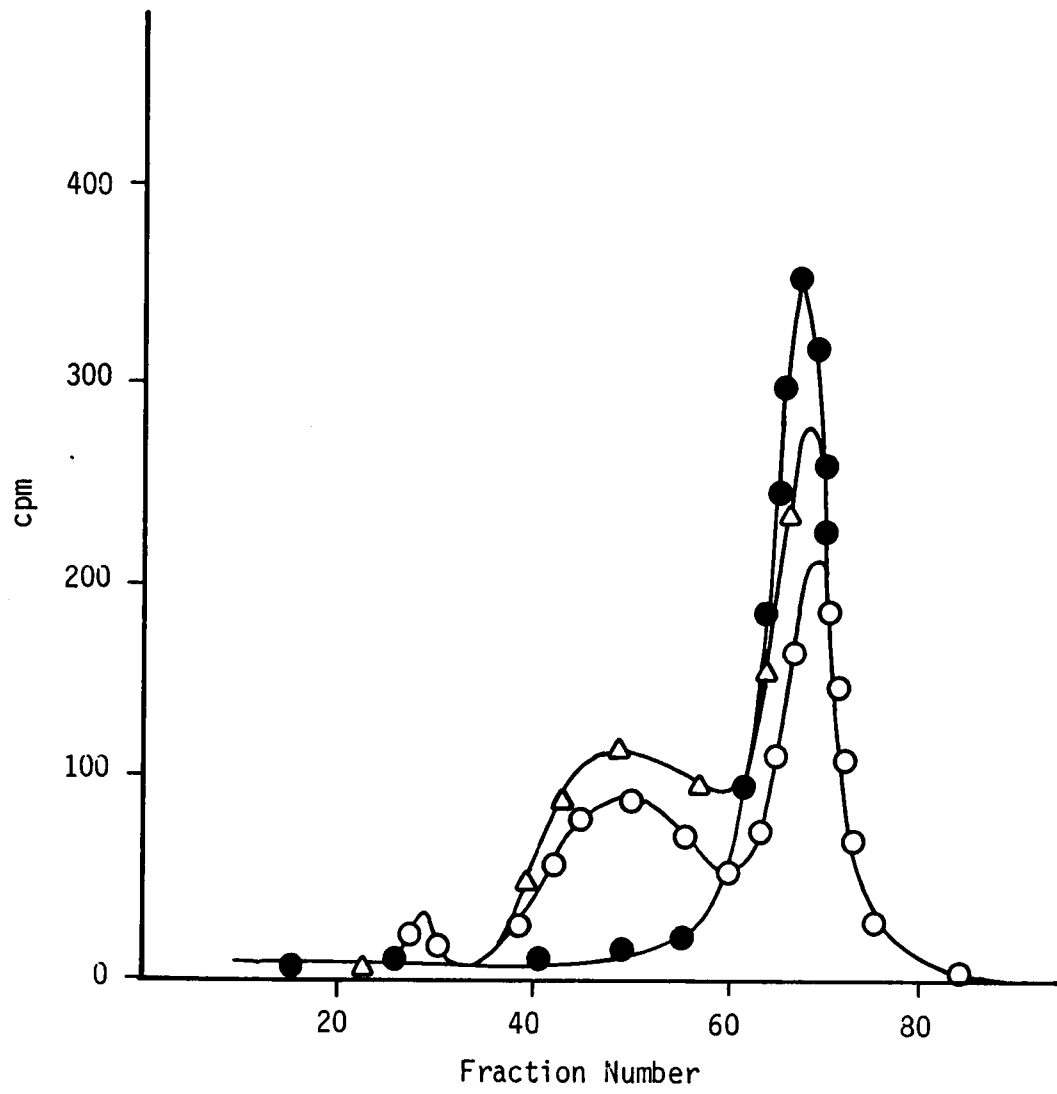


Figure 14



The half-lives for tRNA and rRNA in control and  $\alpha$ -amanitin treated fibroblasts were estimated from the data in Table 7 and from an additional pulse chase experiment in which cells were treated with  $6\mu\text{g/ml}$   $\alpha$ -amanitin. In that experiment ( $6\mu\text{g/ml}$   $\alpha$ -amanitin) the RNAs were purified 40, 52 and 86 hours after removal of the label and the  $\alpha$ -amanitin. The half-lives were calculated from the data in Figure 15.

Transfer RNA turns over with an apparent half-life of 32-42 hours independent of the  $\alpha$ -amanitin concentration. These values for the half-life of tRNA are consistent with values noted by a number of other investigators with a variety of cells.

Ribosomal RNA turns over with a half-life of 56 hours in control cultures, consistent with values noted by other investigators. In  $1\mu\text{g/ml}$   $\alpha$ -amanitin treated cells the half life is 42 hours and 38 hours in cells treated with  $6\mu\text{g/ml}$   $\alpha$ -amanitin. In cells treated with increasing concentrations of  $\alpha$ -amanitin the turnover of rRNA also proportionately increases. That is, "in vivo,"  $\alpha$ -amanitin appears to stimulate the turnover (decrease the half-life) of rRNA but not tRNA. These observations suggest that the in vivo effects of  $\alpha$ -amanitin on rRNA levels may result from increased rRNA turnover as well as decreased synthesis of rRNA when the synthesis of DNA-like RNA is inhibited by low levels of the drug. In support of this conclusion, Sekeris and Schmid (84) have shown the nucleolar RNA polymerase, per se, is not inhibited by  $\alpha$ -amanitin, in vivo, although the incorporation of labeled RNA precursors into isolatable RNA is inhibited. Also, Tata et al. observed that treatment of cells with  $\alpha$ -amanitin resulted in decreased amounts of isolatable nuclear RNA after two to four hours (85).

The specific activities ( $\text{cmp}/A_{260}$ ) for the rRNA and tRNA sized

Figure 15. Effect of  $\alpha$ -amanitin on the turnover of RNA in stimulated fibroblasts. Cells were treated with 1 $\mu$ g/ml, 6 $\mu$ g/ml, 60 $\mu$ g/ml  $\alpha$ -amanitin or not at all, then labeled with [ $^3$ H-CH $_3$ ]-methionine, purified and separated as described in the Experimental Procedures section. rRNA (●—●); tRNA (o—o).

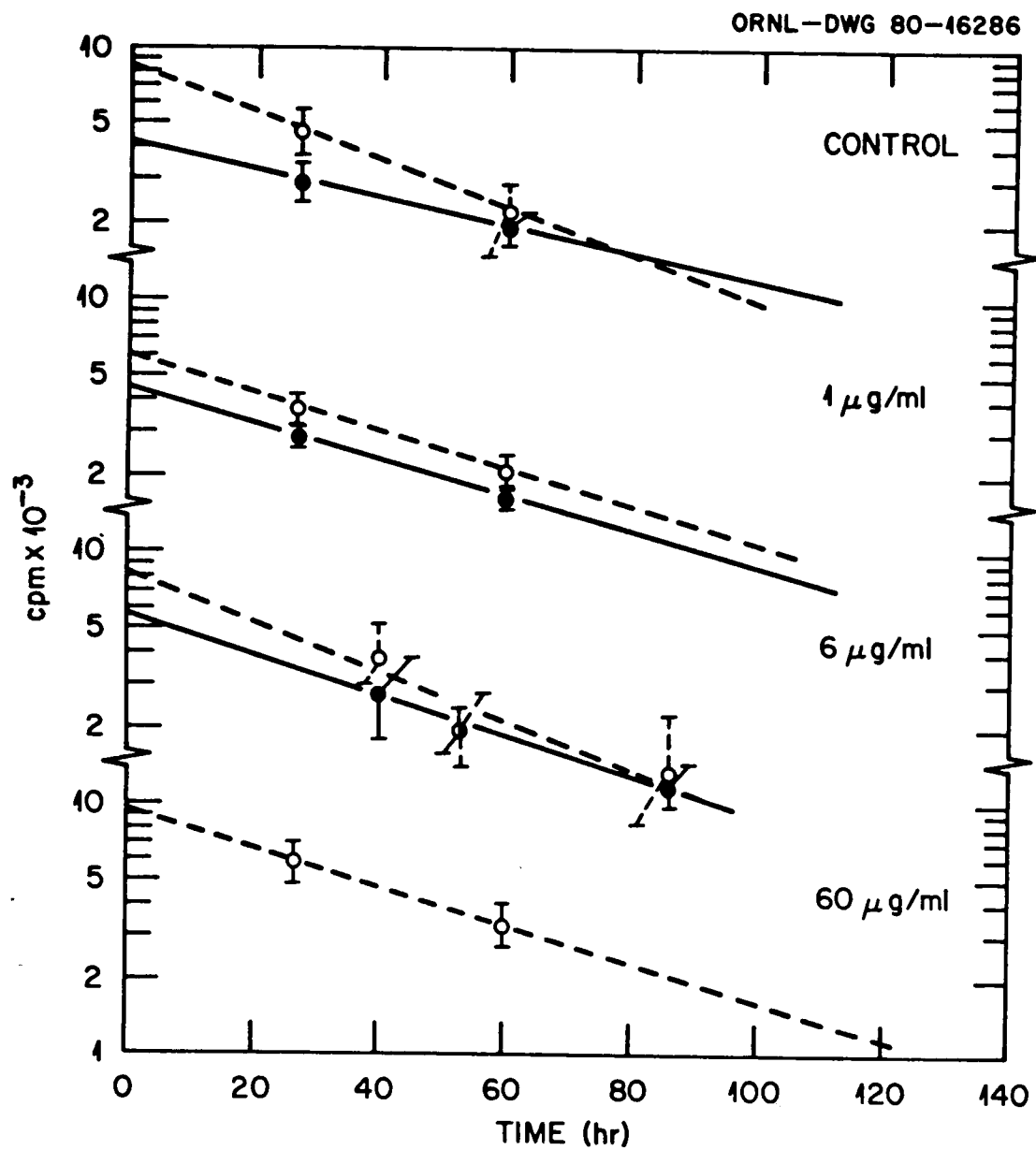


Figure 15

molecules were calculated from the values in Tables 6 and 7. The specific activities (Table 8) are useful in understanding the effects of  $\alpha$ -amanitin on RNA metabolism.

The specific activity of tRNA sized molecules is increased slightly in 1 $\mu$ g/ml treated cells and increased considerably in cells treated with 60 $\mu$ g/ml  $\alpha$ -amanitin. In control as well as  $\alpha$ -amanitin treated cells the specific activity remains constant during the 60 hour recovery period. This indicates there is no additional synthesis of tRNA after the label is removed.

The specific activity of rRNA sized molecules is considerably less than that for tRNA. Also, the specific activity decreases during the 60 hour recovery period, indicating continued synthesis of rRNA during this interval. A decrease in specific activity would also occur if there was selective breakdown of the methionine labeled rRNA molecules. Mechanistically, the processes which would cause selective breakdown of labeled rRNA are not known. The former suggestion is much more likely based on our understanding of the synthesis and breakdown of RNA.

Since the extent of methylation is less than rRNA methylation and the absolute quantity of rRNA is greater than the tRNA quantity, a lower specific activity is not to be unexpected.

Considering the data in Tables 6, 7, and 8 together several conclusions can be drawn on the effects of  $\alpha$ -amanitin on the metabolism of RNA in the cultured fibroblasts.

High levels (60 $\mu$ g/ml) of  $\alpha$ -amanitin inhibit completely the methylation and apparently the synthesis of rRNA as well as stimulate the turnover of rRNA. Lower levels (1 $\mu$ g/ml) do not grossly affect the

TABLE 8  
EFFECT OF  $\alpha$ -AMANITIN ON RNA SPECIFIC ACTIVITY

| $\alpha$ -Amanitin | rRNA              | tRNA   | rRNA     | tRNA   |
|--------------------|-------------------|--------|----------|--------|
|                    | Specific Activity |        |          |        |
|                    | 27 hours          |        | 60 hours |        |
| None               | 889               | 4896   | 480      | 4698   |
| 1 $\mu$ g/ml       | 862               | 6060   | 510      | 5955   |
| 60 $\mu$ g/ml      | ND                | 11,033 | ND       | 11,341 |

synthesis and methylation of rRNA but do appear to increase its turnover.

Decreased levels of tRNA sized molecules (Table 6) are correlated with increased  $\alpha$ -amanitin concentrations. However, there is not a proportional decrease in methylation of these molecules (Tables 6, 7). In fact, there is a considerable increase in the number of methyl groups incorporated/ $A_{260}$  (Table 8). The basis for increased labeling of tRNA sized molecules is not clear from these studies and could be due to one of several reasons.

One possible explanation is that, in fact, the methylation of rRNA is not entirely inhibited by 60 $\mu$ g/ml  $\alpha$ -amanitin. The absence of label in the rRNA region can be explained by assuming there is increased breakdown of newly synthesized (i.e., labeled) rRNA to fragments approximately the size of tRNA in the presence of 60 $\mu$ g/ml  $\alpha$ -amanitin. The intermediate fragments would of necessity have to have a greater number of methylated components per unit length to explain the increased specific activity of the tRNA sized molecules in the 60 $\mu$ g/ml treated cells. This is not an unreasonable assumption, for it is known that methylated components protect nucleic acids from nuclease digestion.

A second possible explanation suggests when the synthesis of rRNA is inhibited by 60 $\mu$ g/ml  $\alpha$ -amanitin the methylation of tRNA is increased. This could be due to an increased number of methylation sites per tRNA or an increased use of radioactive AdoMet relative to unlabeled AdoMet in the methylation of tRNA. In either case the biochemical mechanisms causing such changes are hard for me to conceive. For that reason I favor the former proposal.

As stated previously I have investigated the effects of  $\alpha$ -amanitin

on RNA metabolism to determine if any adverse affects of its use would preclude its use as an agent to investigate the contribution of rRNA to excreted modified nucleosides. Although  $\alpha$ -amanitin is not without its pleiotrophic effects, it can be used at high levels (60 $\mu$ g/ml) to determine if rRNA contributes significantly to the pools of excreted modified RNA components.

Effect of  $\alpha$ -amanitin on modified nucleoside excretion. From the data in Table 7, one would expect that if the excreted nucleosides are derived from turnover of rRNA as well as tRNA, the amount of radioactivity in the excreted nucleosides derived from tRNA should be approximately the same in all cases. The radioactivity in those nucleosides derived from rRNA turnover should be about the same in the control and 1 $\mu$ g/ml treated cells and significantly lower in the 60 $\mu$ g/ml  $\alpha$ -amanitin treated cells. The methylated nucleoside composition data indicates the former (excreted nucleosides derived from tRNA turnover) should be the base and ribose methylated nucleosides and the latter primarily the ribose methylated nucleosides. Thus, I would expect the amount of radioactivity in the base methylated nucleosides to be about equal in all conditions. The level of radioactivity in the ribose methylated nucleosides should be equal in control and 1 $\mu$ g/ml  $\alpha$ -amanitin treated cells and significantly decreased in 60 $\mu$ g/ml  $\alpha$ -amanitin treated cells. However, there would be a finite level of radioactivity in the ribose methylated nucleosides even at 60 $\mu$ g/ml  $\alpha$ -amanitin since tRNA should be a source of these nucleosides as well as rRNA.

Excreted [ $^3\text{H-CH}_3$ ]-methionine labeled nucleosides and bases were

separated on the cation-exchange resin from cells treated with  $1\mu\text{g/ml}$  or  $60\mu\text{g/ml}$   $\alpha$ -amanitin and from control cultures. The media samples were taken 60 hours after removing the radioactive label. The amount of radioactivity in base methylated and ribose methylated nucleosides (Table 9) was determined in duplicate plates. The level of  $\Psi\text{rd}$  (Table 9) was determined by measuring the peak height at 254nm.

Since the base methylated nucleosides ( $m_2^2\text{G}$ ,  $m^2\text{G}$ ,  $m^1\text{G}$ ,  $m^5\text{C}$ ,  $m^7\text{G}$ ,  $m^5\text{U}$ , and  $m^3\text{U}$ ) occur to the greater extent in tRNA, in these cells at least, the amount excreted is not significantly different in control,  $1\mu\text{g/ml}$   $\alpha$ -amanitin or  $60\mu\text{g/ml}$   $\alpha$ -amanitin treated cells. However, when the levels of excreted ribose methylated nucleosides ( $\text{U}_m$ ,  $\text{C}_m$ ,  $\text{G}_m$ ) and  $\Psi\text{rd}$  are compared in the control and treated cells it is quite obvious that when rRNA methylation is inhibited by  $60\mu\text{g/ml}$   $\alpha$ -amanitin the radioactive levels in the media are also significantly decreased. This suggests that the turnover of rRNA contributes to the excretion of modified nucleosides as well as the turnover of tRNA. Since rRNA methylation is not grossly inhibited by  $1\mu\text{g/ml}$   $\alpha$ -amanitin the amount of ribose methylated nucleosides excreted under these conditions is not significantly different from cells which were not treated with  $\alpha$ -amanitin.

It is interesting that there is a significantly greater amount of radioactivity incorporated in the N-5-methylUrd and N-3-methylUrd peak relative to the other methylated nucleosides that are excreted. This is apparently due to the greater labeling of these compounds in rRNA and tRNA (Table 5, page 56).

Although (see RNA compositional data) methylated Urd derivatives are a large percent of the methionine labeled modifications of rRNA there does not appear to be any decrease in the amount excreted when rRNA



TABLE 9  
EFFECT OF  $\alpha$ -AMANITIN ON THE EXCRETION OF MODIFIED NUCLEOSIDES BY STIMULATED FIBROBLASTS<sup>1</sup>

| ( $\alpha$ -Amanitin) | $U_m$             | $C_m$             | $G_m$             | $\psi$            | $m^7GUA$          | $m^2_2G$          | $m^1G$           | $m^2G$           | $m^5C$            | $m^5U/m^3U$            |
|-----------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|------------------|------------------|-------------------|------------------------|
| None                  | 1900 $\pm$<br>375 | 1106 $\pm$<br>303 | 1445 $\pm$<br>280 | 29.6 $\pm$<br>3.8 | 779 $\pm$<br>192  | 638.5 $\pm$<br>25 | 805 $\pm$<br>16  | 437 $\pm$<br>52  | 1136 $\pm$<br>9   | 77,136 $\pm$<br>9,669  |
| 1 $\mu$ g/ml          | 2348 $\pm$<br>292 | 1442 $\pm$<br>24  | 1599 $\pm$<br>342 | 20.5 $\pm$<br>4.0 | 1092 $\pm$<br>136 | 738 $\pm$<br>241  | ND               | 400 $\pm$<br>56  | 1040 $\pm$<br>136 | 80,587 $\pm$<br>20,542 |
| 60 $\mu$ g/ml         | 1291 $\pm$<br>199 | 505 $\pm$<br>112  | 484 $\pm$<br>197  | 20.5 $\pm$<br>2.8 | 918 $\pm$<br>29   | 791 $\pm$<br>132  | 662 $\pm$<br>167 | 585 $\pm$<br>106 | 1078 $\pm$<br>299 | 72,429 $\pm$<br>4,002  |

<sup>1</sup>Cells were treated with  $\alpha$ -amanitin and labeled with [<sup>3</sup>H-CH<sub>3</sub>]-methionine as described in the Experimental Procedures. Nucleosides excreted in the media were purified by silica chromatography and analyzed on the cation-exchange resin. The data presented is an average of two experiments and is summarized as total cpm in 2.5 ml media, except for  $\psi$  where the peak height (nm) was measured. ND = not determined.

synthesis is inhibited. The reason for this is not clear although it may be related to the fact ribothymidine can be salvaged, while the other methylated nucleosides are not. It is not a typical excreted nucleoside in that respect and as a result of this is not as indicative of RNA metabolism as the others are.

When cells are treated with  $1\mu\text{g/ml}$   $\alpha$ -amanitin the excreted methylated nucleosides reveal no decrease in the methylation of rRNA. However, excreted  $\psi\text{rd}$ , a more sensitive measure of rRNA modification indicates a significant decrease of modification at this concentration of  $\alpha$ -amanitin as well as  $60\mu\text{g/ml}$   $\alpha$ -amanitin. Since the total amount of all the methylated components of rRNA is about equal to the number of  $\psi\text{rd}$  residues in rRNA, levels of excreted  $\psi\text{rd}$  should be a more sensitive measure of rRNA modification than levels of individual excreted ribose methylated nucleosides.

A second possible interpretation of these results is the enzyme responsible for  $\psi\text{rd}$  synthesis is short-lived relative to the RNA methylases. Hence, when mRNA synthesis is inhibited by  $1\mu\text{g/ml}$  or  $60\mu\text{g/ml}$   $\alpha$ -amanitin  $\psi\text{rd}$  excretion is decreased since no new  $\psi\text{rd}$  synthetase can be formed to modify the Urd residues of rRNA and tRNA. If the methylases, in general, had a greater lifetime than  $\psi\text{rd}$  synthetase, inhibition of new mRNA synthesis by  $1\mu\text{g/ml}$   $\alpha$ -amanitin would not affect the extent of methylation of RNAs and subsequently the excreted levels of methylated nucleosides.

A third possibility is that excreted  $\psi\text{rd}$  is a measuring of the inhibition of mRNA synthesis and modification at the  $1\mu\text{g/ml}$  drug treatment. There are several facts that argue against this. First,  $\psi\text{rd}$  is not known to be a major modification of mRNA although it is ubiquitous

in rRNA and tRNA. Second, the amount of mRNA in the cell is small relative to the amounts of tRNA and rRNA. Finally, if  $\psi$ rd excretion was decreased due to inhibition of mRNA synthesis one might expect the level of excreted  $m^7\text{Gua}$  to be decreased also since  $m^7\text{Gua}$  is present in the 5'-capped structure common to most mRNAs. However, the amount of excreted  $m^7\text{Gua}$  did not decrease in cells treated with either 1 or 60  $\mu\text{g/ml}$   $\alpha$ -amanitin relative to cells which had no treatment.

Without any information on the stability of the enzymes that modify RNA it is impossible to unequivocally distinguish between the first and second alternatives suggested above. Since the second alternative is based on "proposed" differences in the stability of RNA modification enzymes while the first alternative is based on known facts the first is more credible.

It is my opinion, then, that this data demonstrates that excreted ribose methylated nucleosides and  $\psi$ rd can be derived from the turnover of rRNA as well as tRNA. Also, the excreted base methylated nucleosides are derived from largely tRNA turnover.

Inhibition of RNA synthesis by Actinomycin D. Actinomycin D has been shown to be a selective inhibitor of rRNA synthesis in cultured cells at low concentrations (0.04  $\mu\text{g/ml}$ ) and a general inhibitor of RNA synthesis at much higher (10  $\mu\text{g/ml}$ ) concentrations (89-90). To corroborate the conclusion drawn from the  $\alpha$ -amanitin experiments that both rRNA and tRNA contribute to excreted modified nucleosides, I have inhibited RNA synthesis with Actinomycin D and determined the effect on modified nucleoside excretion.

To confirm that Actinomycin D is a selective inhibitor of rRNA

synthesis at low doses, I have inhibited RNA synthesis and methylation in growing cells with Actinomycin D, purified the RNAs and determined the amount of radioactivity incorporated in the RNAs as described in the Experimental Procedures. Surprisingly, it was observed that low doses of Actinomycin D inhibited tRNA methylation as well as rRNA methylation (Table 10). This is in contrast to observations from other investigators, whom have noted selected inhibition of rRNA synthesis and methylation at low doses of Actinomycin D (89-90).

Since Actinomycin D inhibits tRNA as well as rRNA synthesis in these cells, it is to be expected that the amounts of radioactivity in both the base and ribose methylated nucleosides should be decreased, not just the ribose methylated nucleosides. In fact this is what is observed (Table 11) except for the methylated Urd derivatives peak which was not significantly different in control and Actinomycin D treated cells. Again, this may be related to the salvage of excreted ribothymidine.

These experiments in conjunction with the  $\alpha$ -amanitin experiments demonstrate very clearly that the modified nucleosides which are excreted are derived from the turnover of rRNA as well as tRNA.

The importance of the observation that rRNA as well as tRNA contributes to excreted modified nucleosides is several-fold. First, of main significance, this corrects a previously held misconception of the molecular origin of modified nucleosides that are excreted by cells. Until these experiments, it was previously felt that tRNA alone was the primary source of excreted modified nucleosides (40-47). The ramifications of this new observation suggest to understand the molecular

TABLE 10  
EFFECT OF ACTINOMYCIN D ON [ $^3\text{H-CH}_3$ ]-METHIONINE LABELING  
OF RNA IN SERUM STIMULATED FIBROBLASTS<sup>1</sup>

| Actinomycin D         | tRNA              | rRNA              |
|-----------------------|-------------------|-------------------|
| none                  | 37,840 $\pm$ 2819 | 13,666 $\pm$ 1451 |
| 0.04 $\mu\text{g/ml}$ | 26,312 $\pm$ 2740 | 2,801 $\pm$ 346   |

<sup>1</sup>The data is an average of two independent determinations and the amount of label incorporated was determined 40 hours after removing the label.

TABLE 11  
EFFECT OF ACTINOMYCIN D ON MODIFIED NUCLEOSIDE EXCRETION BY SERUM STIMULATED FIBROBLASTS<sup>1</sup>

| (Actinomycin D) | U <sub>m</sub>  | C <sub>m</sub> | G <sub>m</sub> | ψ            | m <sup>7</sup> Gua | m <sub>2</sub> <sup>2</sup> G | m <sup>1</sup> G | m <sup>2</sup> G | m <sup>5</sup> C | m <sup>5</sup> U/m <sup>3</sup> U |
|-----------------|-----------------|----------------|----------------|--------------|--------------------|-------------------------------|------------------|------------------|------------------|-----------------------------------|
| None            | 10,922±<br>1448 | 4317±<br>549   | 4718±<br>822   | 151±<br>24.9 | 3787±<br>557       | 2944±<br>240                  | 3741             | 2303±<br>148     | 4836±<br>323     | 337,730±<br>112,634               |
| 0.4µg/ml        | 4987±<br>3150   | 1331±<br>18    | 1433±<br>349   | 95±<br>18    | 1674±<br>925       | 2026±<br>206                  | 2147±<br>510     | 509±<br>21       | 2122±<br>155     | 215,836±<br>59,373                |

<sup>1</sup>Cells were treated with Actinomycin D and labeled with [<sup>3</sup>H-CH<sub>3</sub>]-methionine as discussed in the Experimental Procedures. Nucleosides excreted in the media were purified by silica chromatography and analyzed on the cation-exchange resin. The data presented is an average of two experiments and is summarized as total CPM in 10 ml of media, except for ψ where the peak heights (mm) were measured.

basis for increased excretion of modified nucleosides in cancer patients, we must study the metabolism of all classes of RNA, not just tRNA.

## BIBLIOGRAPHY



## BIBLIOGRAPHY

1. Abelson, H. T., et al. 1974. *Cell* 1, 161.
2. Weber, M. J. 1972. *Nature* 235, 58.
3. Eliceiri, G. L. 1976. *BBA* 447, 391.
4. Loeb, J. N., et al. 1965. *Science* 149, 1093.
5. Schlegel, R. A., et al. 1978. *Nuc. Acids Res.* 5, 3715.
6. Liebhaber, S. A., et al. 1978. *Cell* 13, 121.
7. Murphy, W. and Attardi, G. 1973. *PNAS* 70, 115.
8. Lengyel, J. A. and Penman, S. 1977. *Dev. Biol.* 57, 243.
9. Blobel, G. and Potter, V. R. 1968. *BBA* 166, 48.
10. Hanoune, J. and Agarwal, M. K. 1970. *FEBS Lett.* 11, 78.
11. Erdos, T. and Bessada, R. 1966. *BBA* 129, 628.
12. Nomura, Y. 1974. *FEBS Lett.* 45, 223.
13. Altman, S., et al. 1974. *Brookhaven Sym.* p. 26, 12.
14. Mandel, P. 1964. *Prog. Nuc. Acid. Res. and Mol. Biol.* 3, 299.
15. Khym, J. X., et al. 1977. *Anal. Biochem.* 77, 446.
16. Craddock, V. M., et al. 1968. *Biochem. J.* 109, 75.
17. Lipsett, M. H. and Peterkofsky, A. 1966. *P.N.A.S.* 55, 1169.
18. Weiss, S. B. and Legault-Demare, J. 1965. *Science* 149, 429.
19. Starr, J. L. 1962. *BBA* 61, 676.
20. Hayward, R. S. and Weiss, S. B. 1966. *P.N.A.S.* 55, 1161.
21. Fleissner, E. and Borek, E. 1962. *P.N.A.S.* 48, 1199.
22. Fleissner, E. and Borek, E. 1964. *Science* 145, 548.
23. Mandel, L. R., et al. 1966. *Nature* 209, 586.
24. Borek, E. 1963. *C.S.H. Sym. Quan. Biol* 28, 139.

25. Altman, S. 1978. Intl. Rev. Biochem. 17, 37.
26. Zamecnik, P. C. 1971. Can. Res. 31, 716.
27. Transfer RNA. 1977. The MIT Press, Cambridge, MA; Altman, S., ed.
28. Stern, L. and Schulman, L. H. 1978. J. Biol. Chem. 253, 6132.
29. Poldermans, B., et al. 1979. J. Biol. Chem. 254, 909.
30. Agris, P. F. and Soll, D. 1977. In Nucleic Acid and Protein Interactions, Academic Press, New York; Vogel, H. J., ed., pp. 321-344.
31. LiHauer, V. Z. and Inouye, H. 1973. Ann. Rev. Biochem. 42, 439.
32. Kerr, S. J. and Borek, E. 1973. In the Enzymes; Boyer, P. D., ed., Academic Press, New York, pp. 167-195.
33. Nav, F. 1976. Biochimie 58, 629.
34. Gerard, G. F., et al. 1971. Biochem. 10, 1974.
35. Singer, B. and Kroger, M. 1979. Prog. Nuc. Acids Res. and Mol. Biol. 23, 151.
36. Hickey, E. D., et al. 1976. PNAS 73, 19.
37. Roman, R., et al. 1976. Nature 260, 359.
38. Uno, H., et al. 1971. BBA 228, 282.
39. Cheda, G. B. 1971. Purine and Pyrimidine Derivatives Excreted in Human Urine. CRC Handbook of Biochem., 2nd edition, pp. G106-G113.
40. Mandel, L. P., Srinivasan, P. R., and Borek, E. 1966. Nature 209, 586.
41. Shimizo, Mitsuhiro and Fujimura Shinji. 1978. BBA 517, 277.
42. Waalkes, T. P., Gehske, C. W., Zumwalt, R. W., Chang, S. Y., Lakings, D. B., Tormey, D. C., Ahmann, D. L., and Moestel, C. G. 1975. Cancer 36, 390.
43. Cooper, I. A., Wray, G. R., Murphey, T. L. 1974. Europ. J. Cancer 13, 1309.
44. Fink, K. and Adams, W. S. 1968. Arch. Biochem. Biophys. 126, 77.
45. Ahonen, J., Kivisaari, J., Vastamaki, M., Valimaki, M., and Kulouen, E. 1972. BBA 233-238.

46. Borek, E., et al. 1977. Can. Res. 37, 3362.
47. Levine, L., et al. 1975. J. Nat. Can. Inst. 54, 341.
48. Borek, E. and Kerr, S. J. 1972. Adv. Can. Res. 15, 163.
49. Viale, G. L. 1971. Can. Res. 31, 605-608.
50. Craddock, V. M. 1970. Nature 228, 1264-1268.
51. Craddock, V. M. 1969. BBA 195, 351-359.
52. Randerrath, K. 1971. Can. Res. 31, 655-661.
53. Randerrath, E., et al. 1974. Can. Res. 34, 643-653.
54. Randerrath, K. and Randerrath, E. 1969. Anal. Biochem. 28, 110-118.
55. Kuchino, Y. and Borek, E. 1978. Nature 271, 126-129.
56. Okada, N., et al. 1978. PNAS 75, 4247-4251.
57. Kerr, S. J. and Borek, E. 1972. Adv. Enz. 36, 1.
58. Kleiman, L., et al. 1978. Nuc. Acid. Res. 5, 851.
59. Kerr, S. J. and Borek, E. 1972. Adv. Enz. Reg. 2, 63.
60. Hancock, R. L. 1967. Experimenta 23, 806.
61. Maden, B. E. H. and Salim, M. 1974. J. Mol. Bio. 88, 133.
62. Klotwijk, J. and Planta, R. J. 1973. Eur. J. Biochem. 39, 325.
63. Lane, B. G. and Tamaoki, T. 1969. BBA 179, 332.
64. Egawa, K., Choi, Y. C., and Busch, H. 1971. J. Mol. Bio 56, 565.
65. Brown, G. M. and Attardi, G. 1965. BBRC 20, 298.
66. Staehelin, M., et al. 1968. Nature 219, 1363.
67. Ginsberg, T., et al. 1971. Eur. J. Biochem. 21, 249.
68. Iwanami, Y. and Brown, G. M. 1968. Arch. Biochem. Biophys. 124, 472.
69. Desrosiers, R., et al. 1974. PNAS 71, 3971.
70. Munns, T., et al. 1974. Biochem. 13, 4409.
71. Uziel, M. and Selkirk, J. K. 1979. J. Cell Physiol. 99, 217.

72. Chan, T., et al. 1974. J. Cell Physiol. 83, 263.
73. Chan, T. 1973. J. Cell Physiol. 81, 315.
74. Hershfield, M. S., et al.
75. Chan, T. 1979. P.N.A.S. 76, 925.
76. Sabina, R. L., et al. 1979. Molec. Gen. Genet. 173, 31.
77. Roeder, R. G. and Rutter, W. J. 1970. P.N.A.S. 65, 675.
78. Blatti, S. P., et al. 1970. C.S.H.S.Q.B. 35, 649.
79. Zylber, E. A. and Penman, S. 1971. P.N.A.S. 68, 286.
80. Schwartz, L. B., et al. 1974. J. Biol. Chem. 249, 5889.
81. Weinmann, R., et al. 1974. P.N.A.S. 71, 3426.
82. Weinmann, R. and Roeder, R. G. 1974. Fed. Proc. 33, 1349.
83. Weil, P. A. and Blatti, S. P. 1976. Biochem 15, 1500.
84. Sekeris, C. E. and Schmid, W. 1972. FEBS Lett. 27, 41.
85. Tata, L. R., et al. 1972. Nature 238, 161.
86. Jacob, S. T., et al. 1970. B.B.R.C. 40, 334.
87. Weils, D. J., et al. 1979. J. Cell Physiol. 100, 199.
88. Jacob, S. T., et al. 1971. Adv. Enz. Reg. 9, 169.
89. Perry, R. P. and Kelley, D. E. 1970. J. Cell Physiol. 76, 127.
90. Gelbein, H. V., et al. 1974. In Chemical Carcinogenesis, Islo and DiPaolo eds., Marcel Dekker, Inc., New York, pp. 309-351.
91. Wiegiers, V. and Hilz, H. 1971. B.B.R.C. 44, 513.
92. Wiegiers, V. and Kilz, H. 1972. FEBS Lett. 23, 77.
93. Hilz, H., et al. 1975. Eur. J. Biochem. 56, 103.
94. Bross-Bellard, M., et al. 1973. Eur. J. Biochem. 36, 32.
95. Haas, B., et al. 1980. Personal Communication.
96. Bagnasa, A. S. and Finch, L. R. 1972. Anal. Biochem. 45, 24.
97. Khym, J. X. 1975. Clin. Chem. 21, 1245.

98. Uziel, M., et al. 1976. Clin. Chem. 22, 1451.
99. Khym, J. X. 1974. Analytical Ion-Exchange Procedures in Chemistry and Biology, Prentice-Hall, Englewood Cliffs, N. J.
100. Uziel, M., et al. 1968. Anal. Biochem. 25, 77.
101. Uziel, M. and Taylor, S. A. 1978. J. Carbohydrates. Nucleosides. Nucleotides 5, 235.
102. Bandy, A. J. and Cohn, W. E. Unpublished Observations.
103. Uziel, M. and Gassen, H. G. 1969. Biochem. 8, 1643.
104. Mauck, J. C. and Green, H. 1974. Cell 3, 171.
105. Bombik, B. M. and Baserga, R. 1974. P.N.A.S. 71, 2038.
106. Mauck, J. C. and Green, H. 1973. P.N.A.S. 70, 2819.
107. Kaplan, J. G. and Mitchell, M. 1979. In Cell Biology and Immunology of Leukocyte Function, Academic Press, New York, Quastel, ed., pp. 87-106.
108. Riddick, D. H. and Gallo, R. C. 1971. Blood 37, 283.
109. Riddick, A. H. and Gallo, R. C. 1971. Blood 37, 293.
110. Sharma, O. K. and Loeb, L. A. 1973. B.B.R.C. 50, 172.
111. Wilkinson, C. R. and Kerr, S. J. 1979. J. Cell Physiol. 98, 395.
112. Friedkin, M. and Kornberg, A. 1957. In the Chemical Bases of Heredity, McElroy and Glass, eds., John Hopkins Press, Baltimore, pp. 609-614.
113. Treswyn, R. W. and Kerr, S. J. 1968. Can. Res. 38, 2285.
114. Culp, L. A. and Brown, G. M. 1968. Arch. Biochem. Biophys. 124, 483.
115. Stout, R. W., et al. 1976. Anal. Biochem. 76, 342.
116. Daddona, P. E., et al. 1980. J. Biol. Chem. 255, 5681.
117. Lothrop, C. D. and Uziel, M. 1980. Clin. Chem. 26, 1430.
118. Lothrop, C. D. and Uziel, M. 1980. Anal. Biochem. 109, 160.
119. Morris, C. J. O. R. and Morris, P. 1976. Separation Methods in Biochemistry, John Wiley and Sons, New York, pp. 155-248.

120. Khym, J. X. 1967. Methods in Enz. 12A, 93.
121. Mandel, L. R. and Borek, E. 1961. B.B.R.C. 6, 138.
122. Nielsen, H. R., et al. 1974. Can. Res. 34, 1381.
123. Nielsen, H. R., et al. 1972. Acta Pathol. Microbiol. Scand. 80, 687.
124. Hoffee, P. A. and Jones, M. E. 1978. Methods in Enz. 51, entire volume.

## APPENDIXES

APPENDIX A

PREPARATIVE CHROMATOGRAPHY OF NUCLEIC ACID COMPONENTS ON SILICA



A number of low molecular weight components including the amino acids, vitamins and nucleic acid components are present in the soluble fractions from cells or culture media. The nucleic acid components and certain vitamins are the major UV absorbing low molecular weight compounds. Since there are a number of compounds in the soluble fractions that interfere with subsequent quantitative analysis, a specific purification step is needed prior to separation of the nucleic acid components on an analytical scale. In addition, this problem is compounded by the fact that nucleosides, bases and nucleotides have very similar physical properties. Due to this, the chromatographic separation of these compounds is somewhat limited without prior fractionation.

The procedure of Uziel et al. (71, 98) was used to concentrate nucleosides from the media. This purification step was adequate for separating nucleosides from the culture media, but there was no separation of bases, 2-O-methyl nucleosides, and deoxynucleosides from nucleotides.

The ready availability of prepacked silica cartridges prompted me to see if they could be used to simplify the analysis of acid-soluble pools from cell or tissue extracts. The technique (117-118) not only has proven to reduce significantly the time for sample preparation, but also is considerably simpler and more precise than other published methods for group separation. To the best of my knowledge this is the first use of borate-impregnated silica for the separation of nucleic acid components. However, Morris has studied the separation of polyhydroxy fatty acids on silica adsorbents impregnated with borate or arsenite (119).

To determine optimum conditions for separating nucleotides from bases and nucleosides, mixtures of each were chromatographed at several different acetonitrile concentrations on the silica cartridges. These results from these experiments are recorded in Tables 12, 13, and 14 for bases, nucleosides, and nucleotides, respectively. The results indicate that greater than 90% of the bases and nucleosides do not bind to the silica gel when applied in a solution that is 90% or less acetonitrile ( $\text{CH}_3\text{CN}$ ). When a sample is adjusted to 95% (v/v) acetonitrile and chromatographed on a silica gel, a significant amount of the more polar nucleosides (cytidine and guanosine) is bound to the gel. Less polar nucleosides (adenosine, uridine, and ribothymidine) and bases are not significantly bound to the gel under these conditions. Greater than 90% of the nucleotides bind to the silica gel when samples are adjusted to 90 or 95% acetonitrile and chromatographed on cartridges equilibrated with these solutions. However, nucleotides are not bound quantitatively to a gel equilibrated with 85% acetonitrile.

It is well established that nucleosides can form a stable complex with borate, at alkaline pH (120); and because no separation of nucleosides from bases is obtained by silica chromatography under the conditions described to this point, I have investigated the effect of including ammonium borate in the elution buffer on nucleoside retention. The complex formed between ribonucleosides and borate has a formal negative charge. If a complex could be formed in the presence of acetonitrile, the retention of nucleosides on silica should be significantly increased and, in fact, this is what is observed (Figure 16). By washing the gel with 0.05 M HAc in 90%  $\text{CH}_3\text{CN}$ , I was then able to selectively elute the nucleosides since the complex is not stable at acidic pH.

TABLE 12  
EFFECT OF ACETONITRILE ON ADSORPTION OF BASES ON SILICA<sup>1</sup>

| Solvent composition,<br>acetonitrile/water | % of total bases recovered |       |      |       |
|--------------------------------------------|----------------------------|-------|------|-------|
|                                            | Cyt                        |       | Ura  |       |
|                                            | Wash                       | Bound | Wash | Bound |
| 85/15 <sup>2</sup>                         | 100                        | ND    | 100  | ND    |
| 90/10 <sup>2</sup>                         | 98                         | 2     | 100  | ND    |
| 90/10 <sup>3</sup>                         | 98                         | 2     | 99   | 1     |
| 95/5 <sup>3</sup>                          | 96                         | 4     | 100  | ND    |

<sup>1</sup>Nonabsorbed fractions (acetonitrile/water wash) and bound fractions (water wash) were concentrated by evaporation, resuspended in less than 100  $\mu$ l of water, and chromatographed on the anion-exchange resin to identify the ultraviolet-absorbing material.

<sup>2</sup>10-ml wash after sample application.

<sup>3</sup>15-ml wash after sample application.

ND = nondetectable

TABLE 13  
EFFECT OF ACETONITRILE ON ADSORPTION OF NUCLEOSIDES ON SILICA<sup>1</sup>

| Acetonitrile/<br>water | % of total nucleosides recovered |       |    |      |       |    |      |       |    |      |       |    |
|------------------------|----------------------------------|-------|----|------|-------|----|------|-------|----|------|-------|----|
|                        | 5-Me-Urd                         |       |    | Urd  |       |    | Ado  |       |    | Guo  |       |    |
|                        | wash                             | bound | ND | wash | bound | ND | wash | bound | ND | wash | bound | ND |
| 85/15 <sup>2</sup>     | 100                              | ND    | ND | 100  | ND    | ND | 100  | ND    | ND | 100  | ND    | 96 |
| 90/10 <sup>2</sup>     | 100                              | ND    | ND | 100  | ND    | ND | 100  | ND    | ND | 100  | ND    | 92 |
| 90/10 <sup>3</sup>     | 100                              | ND    | ND | 100  | ND    | ND | 100  | ND    | ND | 100  | ND    | 93 |
| 95/5 <sup>3</sup>      | 96                               | 4     | 5  | 95   | 5     | 6  | 94   | 6     | 81 | 19   | 37    | 63 |

<sup>1</sup> Nonadsorbed fractions (acetonitrile/water wash) and bound fractions (water wash) were concentrated by evaporation, resuspended in less than 100 µl of water, and chromatographed on the anion-exchange resin to identify the ultraviolet-absorbing material.

<sup>2</sup> 10-ml wash after sample application

<sup>3</sup> 15-ml wash after sample application

ND = nondetectable

TABLE 14

EFFECT OF ACETONITRILE ON ADSORPTION OF NUCLEOTIDES ON SILICA<sup>1</sup>

| Acetonitrile/<br>water | % of total nucleotides recovered |       |  |      |       |  |      |       |  |      |       |  |
|------------------------|----------------------------------|-------|--|------|-------|--|------|-------|--|------|-------|--|
|                        | CMP                              |       |  | UMP  |       |  | AMP  |       |  | CMP  |       |  |
|                        | wash                             | bound |  | wash | bound |  | wash | bound |  | wash | bound |  |
| 85/15 <sup>2</sup>     | 6                                | 94    |  | 70   | 30    |  | 22   | 78    |  | ND   | 100   |  |
| 90/10 <sup>2</sup>     | ND                               | 100   |  | ND   | 100   |  | 1    | 99    |  | ND   | 100   |  |
| 90/10 <sup>3</sup>     | ND                               | 100   |  | ND   | 100   |  | 11   | 89    |  | ND   | 100   |  |
| 95/5 <sup>3</sup>      | ND                               | 100   |  | ND   | 100   |  | ND   | 100   |  | ND   | 100   |  |
|                        |                                  |       |  |      |       |  |      |       |  | 6    | 94    |  |
|                        |                                  |       |  |      |       |  |      |       |  | ND   |       |  |
|                        |                                  |       |  |      |       |  |      |       |  | 5    | 95    |  |
|                        |                                  |       |  |      |       |  |      |       |  | 7    | 93    |  |
|                        |                                  |       |  |      |       |  |      |       |  | 6    | 94    |  |

<sup>1</sup>Nonadsorbed fractions (acetonitrile/water wash) and bound fractions (water wash) were concentrated by evaporation, resuspended in less than 100  $\mu$ l water, and chromatographed on the anion-exchange resin to identify the ultraviolet-absorbing material.

<sup>2</sup>10-ml wash after sample application.

<sup>3</sup>15-ml wash after sample application.

ND = nondetectable.

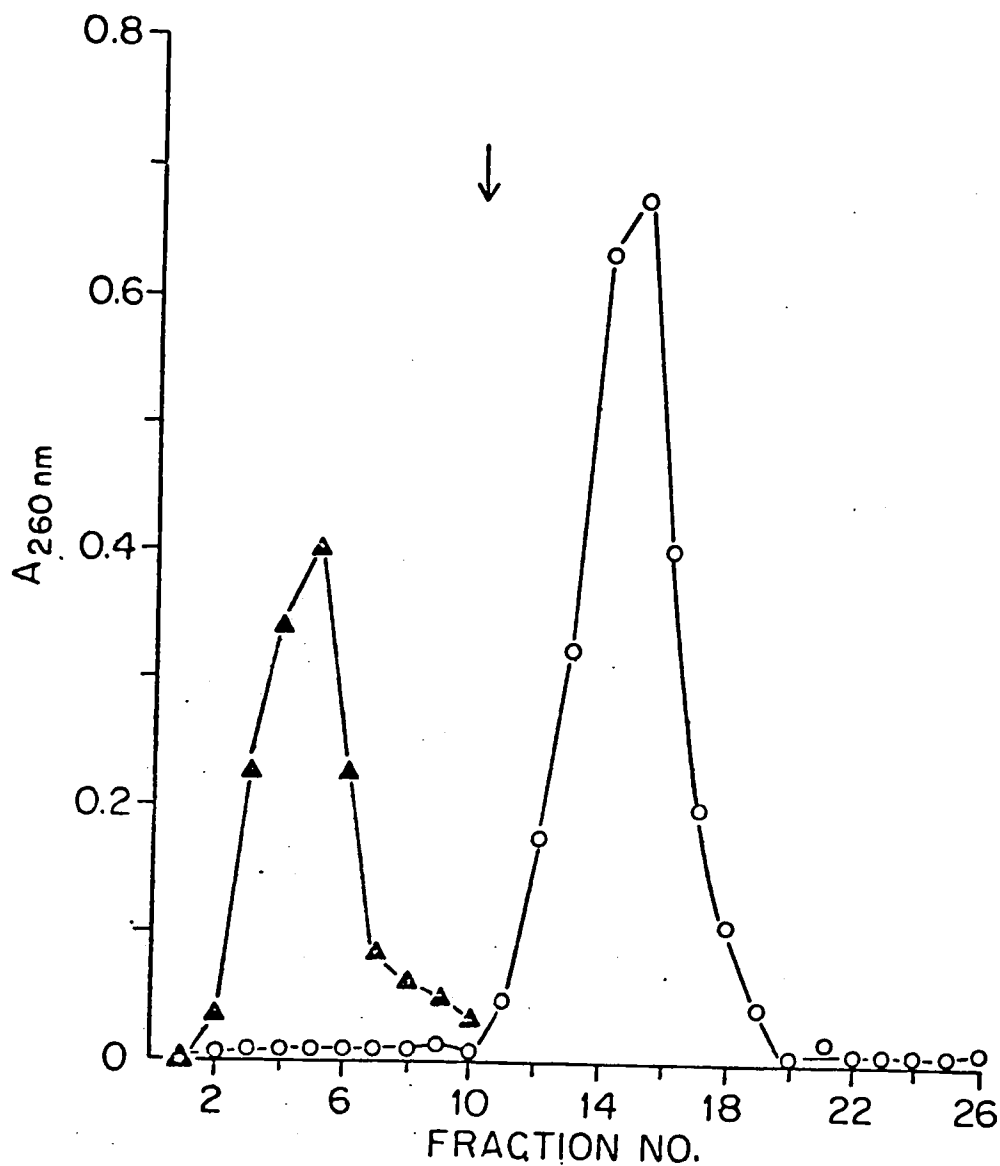


Figure 16. Effect of ammonium borate on nucleoside retention on silica cartridges. A mixture of nucleosides was chromatographed on a silica cartridge that had been equilibrated (o) or had not been ( $\Delta$ ) equilibrated with ammonium borate. One ml fractions were collected and the absorbance at 260 nm, measured for each fraction.

Since it was evident that inclusion of ammonium borate in the buffer did significantly affect the retention of nucleosides, I reinvestigated the effect of  $\text{CH}_3\text{CN}$  plus borate on the efficiency of group separations. Cytosine (Cyt), uridine (Urd), cytidine (Cyd), and uridine 5'-monophosphate (UMP) were chosen as models for their respective groups because they are the most difficult to separate from each other. UMP is the least tightly bound nucleotide; Cyt is bound most tightly of all the major bases; and Urd and Cyd are the least and most tightly bound of the major nucleosides, respectively. The frontal elution technique that can separate the Cyd/UMP pair and the Cyt/Urd pair will also separate the remaining bases, nucleosides or nucleotides from each other.

Mixtures of Cyd and UMP or Cyd, Cyt, and UMP were chromatographed on silica gels at several acetonitrile concentrations, then were concentrated and subsequently chromatographed on the anion-exchange resin to confirm the identity of the UV-absorbing material. The results (Table 15) from these experiments agree with the preliminary experiments; separations are obtained when gels are equilibrated and nucleosides are eluted with 90% acetonitrile. However, in addition, bases can be separated from nucleosides as well as nucleotides.

Acetic acid was added to the 90% acetonitrile to decompose the borate-nucleoside complex and allow elution of the nucleosides. Mixtures of Cyd, Urd, and UMP were used to determine the optimum concentration of acetic acid for elution of the nucleoside group (Table 16). The results show that the acetic acid concentration should be 0.01 M or greater to ensure complete elution of nucleosides before the nucleotides are eluted with water. At the acetic acid concentrations tested

TABLE 15  
EFFECT OF ACETONITRILE ON EFFICIENCY OF GROUP SEPARATIONS<sup>1</sup>

| Percent acetonitrile | Compound | % of total recovered |            |                 |
|----------------------|----------|----------------------|------------|-----------------|
|                      |          | Base                 | Nucleoside | Nucleotide      |
| 70                   | Cyd      | 92                   | 8          | ND <sup>b</sup> |
|                      | UMP      | 91                   | 9          | ND              |
| 80                   | Cyd      | 69                   | 27         | 4               |
|                      | UMP      | 92                   | 8          | ND              |
| 85                   | Cyd      | 19                   | 80         | 1               |
|                      | UMP      | 14                   | 36         | 50              |
| 90                   | Cyt      | 83                   | 11         | 6               |
|                      | CYD      | 2                    | 91         | 7               |
|                      | UMP      | ND                   | ND         | 100             |
| 100                  | Cyd      | 2                    | ND         | 98              |
|                      | UMP      | 68                   | ND         | 32              |

<sup>1</sup>Mixtures of Cyd and UMP, or Cyt, Cyd, and UMP were chromatographed over new silica gels at various concentrations of acetonitrile. Columns were washed first with 5 ml of 4 mM ammonium borate, then with 5 ml of 4 mM ammonium borate in 70, 80, 85, 90, or 100% acetonitrile in water. Samples were applied in 3 ml of the initial solvent, and gels washed with an additional 7 ml of this solvent. Columns were next washed with 16 ml of 0.05M acetic acid in 70, 80, 85, 90, or 100% acetonitrile in water, and finally with 9 ml of water. Groups (bases, nucleosides, or nucleotides) were concentrated by evaporation and subsequently analyzed on the anion-exchange resin.

ND = nondetectable.



TABLE 16  
EFFECT OF ACETIC ACID ON NUCLEOSIDE AND NUCLEOTIDE SEPARATIONS<sup>1</sup>

| Acetic acid<br>concentration (M) | Compound | Base            | Nucleoside | Nucleotide |
|----------------------------------|----------|-----------------|------------|------------|
| 0.001                            | Urd      | 7               | 83         | 10         |
|                                  | Cyd      | ND <sup>b</sup> | 69         | 31         |
|                                  | UMP      | ND              | ND         | 100        |
| 0.005                            | Urd      | 3               | 92         | 5          |
|                                  | Cyd      | ND              | 90         | 10         |
|                                  | UMP      | ND              | 5          | 95         |
| 0.01                             | Urd      | 8               | 92         | ND         |
|                                  | Cyd      | ND              | 97         | 3          |
|                                  | UMP      | ND              | 1          | 99         |
| 0.05                             | UMP      | ND              | ND         | 100        |
| 0.1                              | UMP      | ND              | 1          | 99         |
| 1.0                              | UMP      | ND              | 1          | 99         |

<sup>1</sup>Mixtures of Urd, Cyd, and/or UMP were chromatographed on new silica gels at various acetic acid concentrations in the nucleoside elution buffer. Gels were first washed with 5 ml of 4 mM ammonium borate, then with 5 ml of 4 mM ammonium borate in 90% acetonitrile. Samples were applied in 3 ml of the equilibration buffer, and gels were washed with an additional 7 ml of this buffer. Gels were then washed with 16 ml of 90% acetonitrile which was also 0.001, 0.005, 0.01, 0.05, 0.1, or 1M acetic acid, and finally with 9 ml of water.

ND = nondetectable

(0.001-1 M), there was no early elution of nucleotides before the silica gel was washed with water.

### Quantitation and Capacity of Silica Gels

Using the scheme suggested in the experimental procedures, I have applied several compounds to a silica gel and quantitated the recovery of each compound (Table 17). The mean recovery in these experiments was 100% with a standard deviation of  $\pm 4.4\%$  ( $n = 3$ ). The capacities of the silica gels were determined by chromatography of Cyd solutions (2.5 - 6.3 mM) several times on new silica gels. The capacity of each gel ( $n = 4$ ) was calculated (98); it ranged from 48 to 78  $\mu\text{mol/ml}$  silica. The mean of the calculated capacities was  $61 \pm 13 \mu\text{mol Cyd/ml}$  silica. Due to the inherent high capacity of the silica gels, acid-soluble pools from a large number of cells ( $10^7 - 10^9$ ) can be prepared on a single column.

A summary of retention volume data for numerous chromatographs on the silica gels is given in Tables 18 and 19. I have illustrated the data as bars of length  $2W$ , where  $W$  is the peak width at  $1/2$  peak height. The center of each bar is the retention volume. The data from these experiments have allowed me to speculate on the nature of the retention mechanisms for nucleic acid components on silica gel. I have chromatographed charged, as well as neutral, compounds on the silica gels. Nucleosides and nucleotides that are charged, either positive, negative, or zwitterionic, are strongly adsorbed to silica. Compounds which have no charge at neutral pH are not retained. The negatively charged nucleoside-borate complex is more soluble in the silica phase. Complexed nucleosides are then separated from bases, 2'-O-methyl nucleosides,

TABLE 17  
QUANTITATION OF SILICA CHROMATOGRAPHY<sup>1</sup>

| Compound | A <sub>260</sub><br>applied | A <sub>260</sub><br>recovered | Percent<br>recovery |
|----------|-----------------------------|-------------------------------|---------------------|
| 5-MeUrd  | 1.22                        | 1.28                          | 105                 |
| Cyd      | 1.94                        | 1.88                          | 97                  |
| 5'-GMP   | 0.393                       | 0.387                         | 98                  |

<sup>1</sup>Cyd, N-5-Me Urd, and 5'-GMP were chromatographed individually on a new silica gel in the three step fractionation procedure. For each sample, the absorbance at 260 nm was measured before and after silica chromatography. The number of absorbance units was calculated as the product of the volume times the absorbance of the solution, as measured with a one cm light path. The percent recovery was calculated as the absorbance recovered times 100 divided by the absorbance applied.

TABLE 18  
RETENTION VOLUME FOR METHYLATED NUCLEOSIDES  
ON SILICA

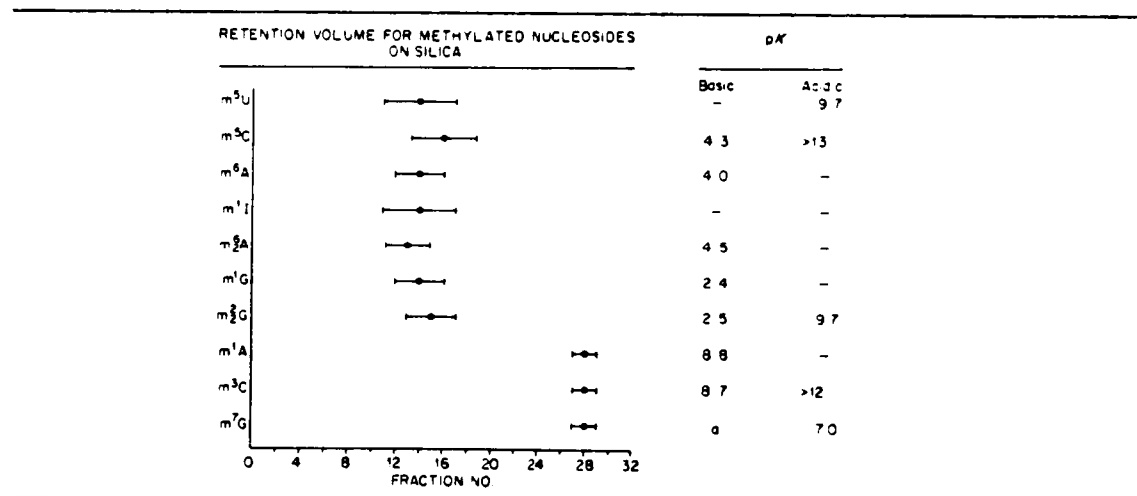
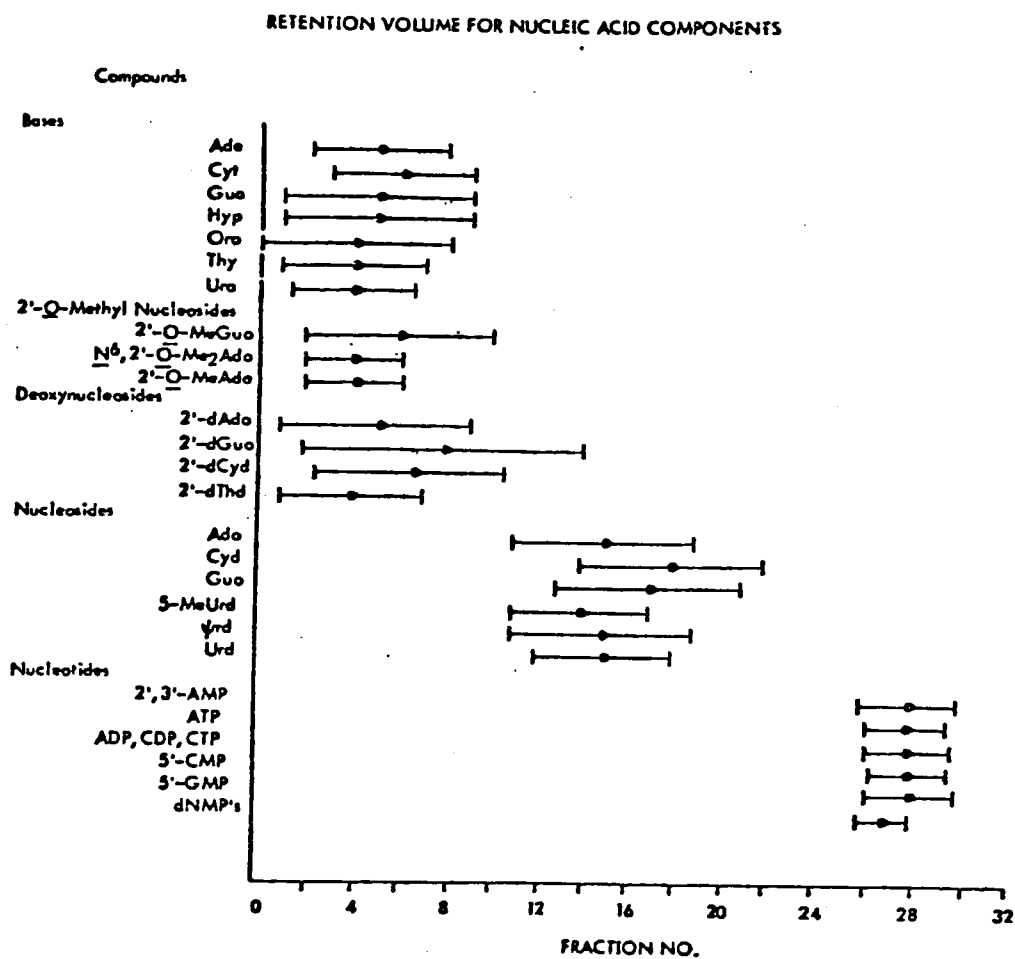


TABLE 19  
RETENTION VOLUMES FOR NUCLEIC ACID COMPONENTS ON SILICA



<sup>1</sup> Authentic compounds individually or as mixtures were chromatographed over several silica gels. Columns were washed and samples eluted as described in the Experimental Procedures. Fractions (1 ml) were collected, and the absorbance was measured in a DU spectrophotometer after each fraction was diluted with 2 ml of water. The data is summarized as described in the text.

and deoxynucleosides, none of which can complex with borate. Cationic nucleosides separate from nucleosides with no charge and chromatograph like negatively charged nucleotides on the silica gels. The observation that charged compounds, irrespective of the nature of the charge, bind to silica gel suggests that ion exchange is not the primary mode of retention. If ion exchange were the primary mode of retention, either positive or negative compounds would bind to the gel, but not both. As discussed by Morris and Morris in a recent review (119), it is now generally agreed that hydrogen bonding to surface silanol groups is the basis for retention on silica absorbants. My observations are consistent with this proposal.

In short, these experiments can be summarized in the following scheme for optimum performance:

Procedures found to give optimum separations on the silica gels were:

1. Prewash columns with 5 ml of 0.4 mM ammonium borate, then wash with 5 ml of equilibration buffer.
2. Load samples in 3 ml or less of equilibration buffer and wash gels with an additional 10 ml. Bases, 2'-O-methyl nucleosides, and deoxynucleosides elute in this wash. For quantitative elution of Urd and other loosely bound nucleosides in the acetic acid buffer, wash gels with less than 7 ml of equilibration buffer following sample application.
3. Wash columns with 16 ml of 0.05 M acetic acid in 90% acetonitrile. Nucleosides elute with this wash.
4. Wash columns with 9 ml of water to elute nucleotides and charged nucleosides.

Thus, prior to the analysis, by ion-exchange chromatography, of the nucleic acid components excreted in the culture media or in acid extracts of cells, I have fractionated the nucleic acid components in the soluble pools by silica chromatography (117-118), or have purified the nucleosides by affinity absorption (98).

## APPENDIX B

### SALVAGE OF RIBOTHYMIDINE FROM RNA TURNOVER IN CULTURED FIBROBLASTS

In the de novo synthesis of thymidylate the N-5-methyl group is donated by the cofactor, folate (112). In the synthesis of ribothymidine in RNA Adenomet is the methyl donating cofactor rather than folate (121). However, Nielson et al. have noted that [ $^3\text{H-CH}_3$ ]-methionine labels thymine as well as N-5-MeCyt in DNA (122-123). It is well-established that the methyl moiety of N-5-MeCyt is derived from Adenomet. That [ $^3\text{H-CH}_3$ ]-methionine labels thymine in DNA was unexpected since folate, not Adenomet, is the methyl donating cofactor for the DNA precursor thymidylate. The basis for methionine labeling of thymine is not clear for there are several possible mechanisms:

1. Deamination of N-5-MeCyt on macromolecular DNA.
2. Salvage and incorporation during DNA synthesis of ribothymidine liberated from RNA turnover.
3. Both (1) and (2).

Methionine labeling of DNA was observed in the course of my experiments, also. However, I have not digested the DNA to see if the label was in N-5-methyl Cyt, thymidine, or both. The amount of label in DNA was only 1-2% of the radioactivity in RNA + DNA. However, extensive studies were made on the soluble pools from cells as well as the excreted nucleic acid metabolites. The observations discussed below suggest that a salvage pathway for ribothymidine (option 2 above) is active in these cells. Several pathways (Figure 17) for conversion of ribothymidine to deoxythymidylate are possible (124).

It is known that Urd kinase will phosphorylate rThd to form rTMP which upon subsequent phosphorylation to rTDP and rTTP ribonucleotide reductase would reduce to dTDP and dTTP (124). Alternatively rThd



Figure 17. Salvage pathways for conversion of ribothymidine from RNA to deoxythymidylate. The pathways were hypothesized from known enzymes and their substrate specificities (124).

ORNL-DWG 80-16289

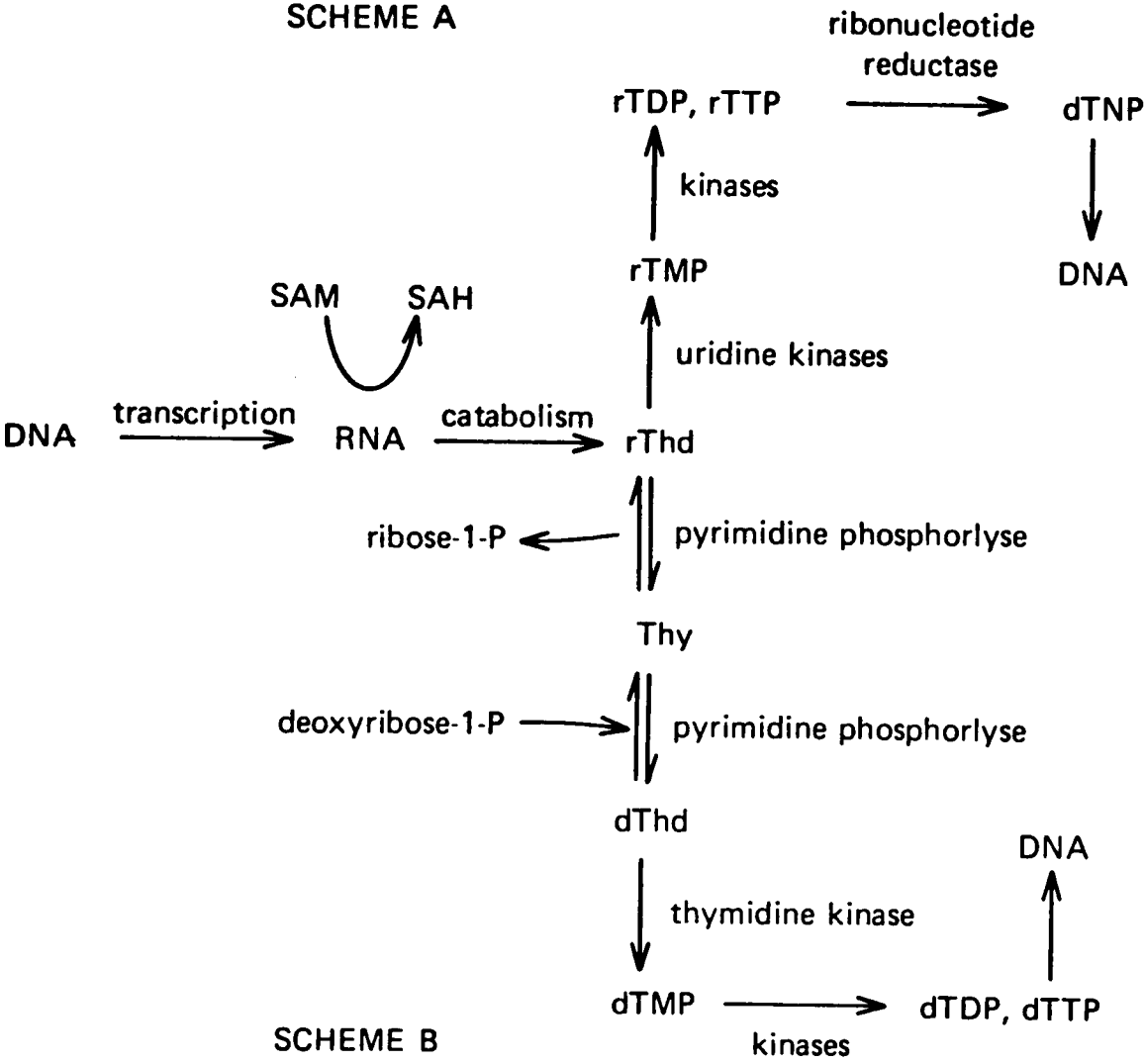


Figure 17

could be phosphorylyzed to Thy + ribose 1P. This reaction is known to be readily reversible under physiological conditions and in the presence of deoxyribose-1P, thymidine would be produced and subsequently phosphorylated to dTNP (124).

[ $^3\text{H-CH}_3$ ]-methionine labeled dThd and rThd are excreted by the fibroblasts (Figures 5, 12) while radioactive rThd and dThd are found in the acid soluble pools (Figures 18, 19) in these cells.

The nucleotide pools, after methionine labeling of the cells, were extracted, separated from contaminants by silica chromatography, and digested with alkaline phosphatase. The resulting nucleosides were fractionated into deoxy and ribonucleoside groups by a second pass on the silica cartridge. An aliquot of each group was then chromatographed on the cation-exchange resin.

The only [ $^3\text{H-CH}_3$ ]-methionine labeled ribonucleoside recovered from the alkaline phosphatase digestion of the nucleotides was ribothymidine (Figure 20), although several other methionine labeled ribonucleosides are present in the soluble pools (Figure 18). This observation indicates ribothymidine was present in the soluble pools as rTXP as well as rThd. In addition, [ $^3\text{H-CH}_3$ ]-methionine labeled dThd was recovered from the alkaline phosphatase digests of the nucleotide pools (Figure 21). This indicates the presence of methionine labeled dTXP in the acid-soluble pools. Thus, most of the intermediates (rThd, dThd, dTNP) of scheme B can be isolated from cells and/or media while all of the metabolites from scheme A can be isolated.

These experiments suggest that several pathways for salvage of ribothymidine from RNA exist. This conclusion is based on the fact that several intermediates in these pathways are isolated from either

Figure 18. [ $^3\text{H-CH}_3$ ]-methionine labeled ribonucleosides in acid extracts of cultured fibroblasts. The acid-soluble pools were extracted and fractionated into groups by silica chromatography as described in the Experimental Procedures section. The nucleoside group after concentration by evaporation was applied directly to the cation-exchange resin.

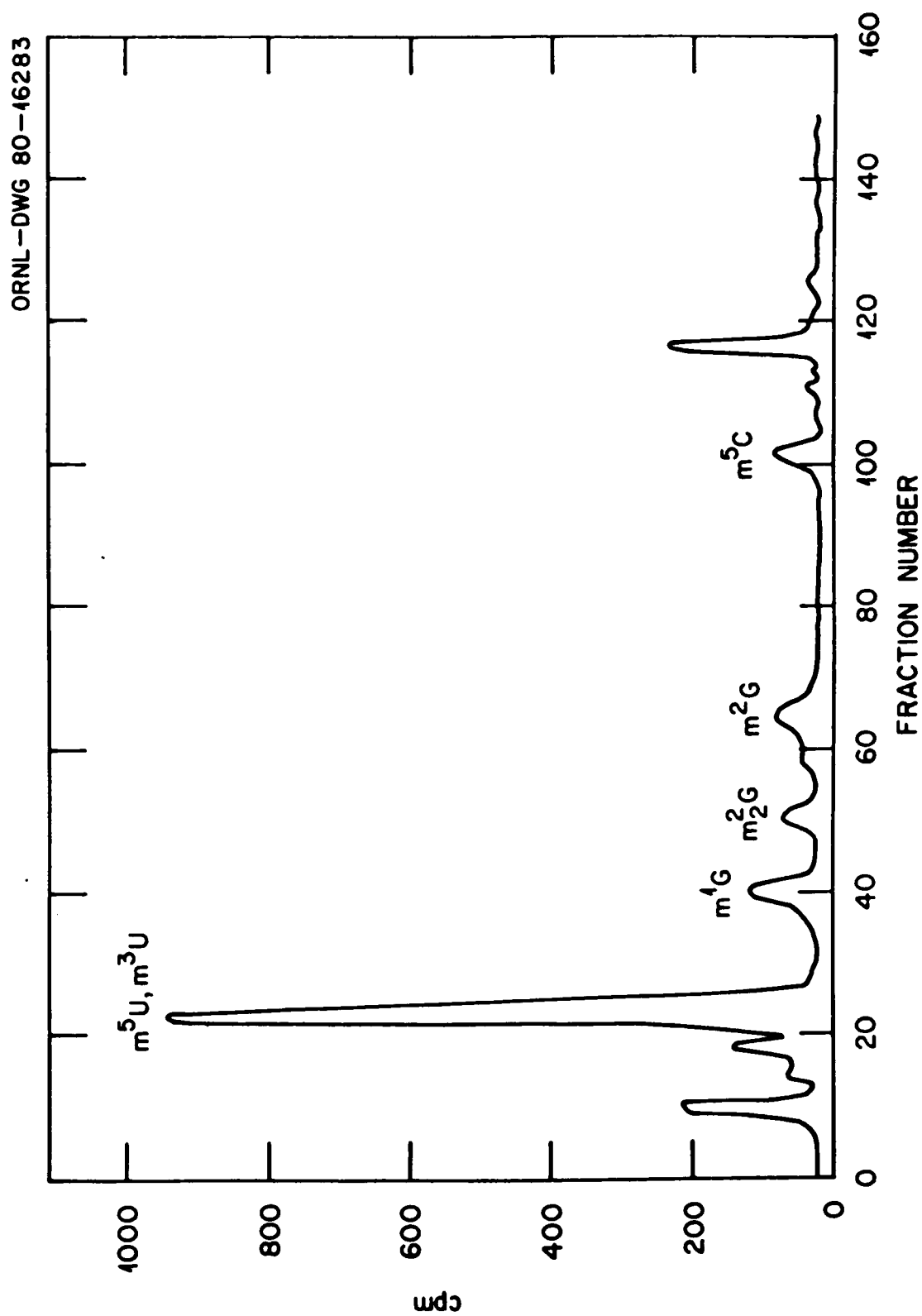


Figure 18

Figure 19. [ $^3\text{H-CH}_3$ ]-methionine labeled ribose methylated and deoxynucleosides in acid extracts of cultured fibroblasts. The soluble pools were prepared and chromatographed as in the legend to Figure 18, except that the ribose-methylated and deoxynucleoside group was applied to the cation-exchange resin.

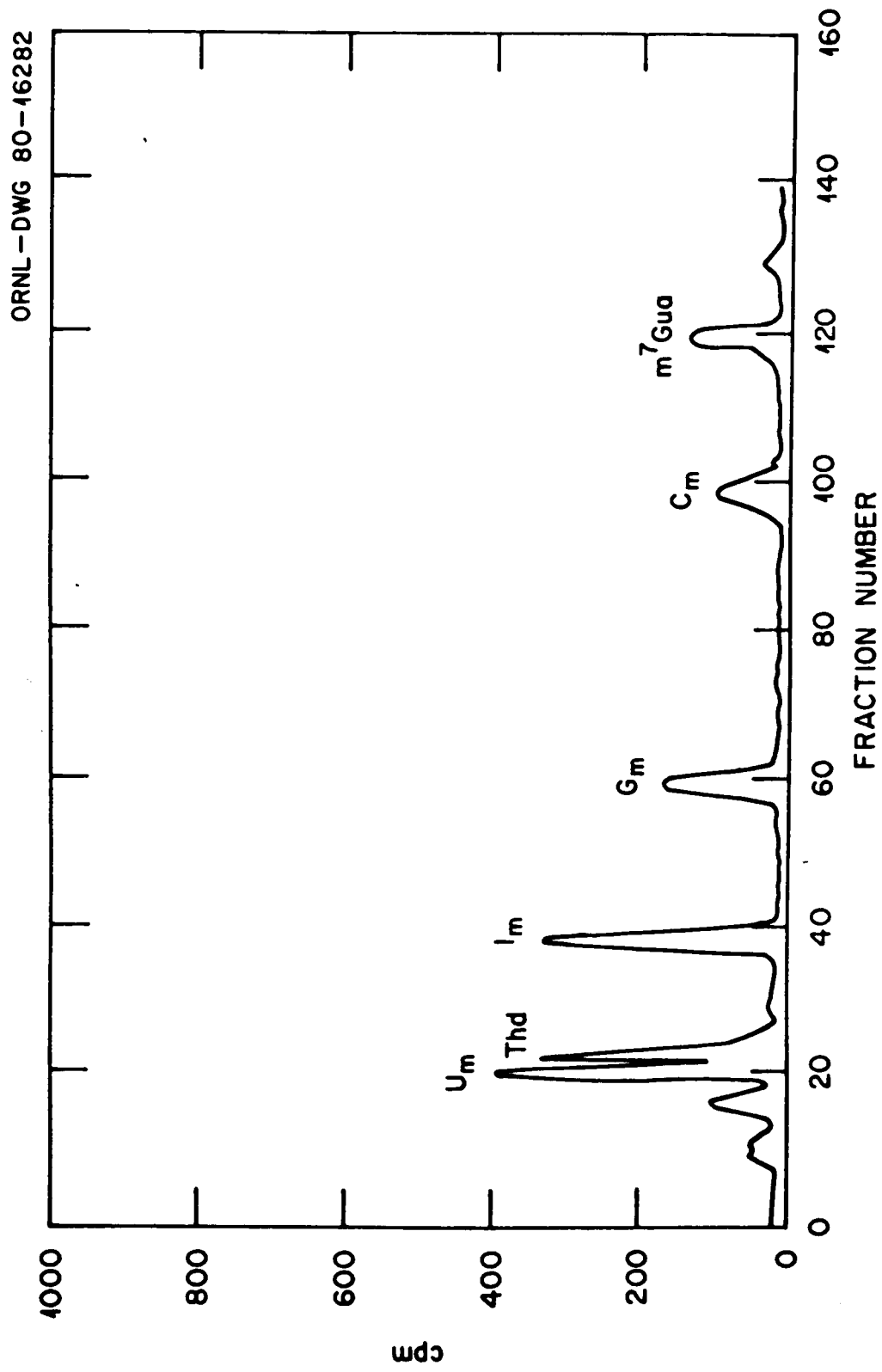


Figure 19

Figure 20. [ $^3\text{H-CH}_3$ ]-methionine labeled ribonucleosides from alkaline phosphatase digestion of acid soluble nucleotides. The nucleotide pools were prepared from the acid extracts by silica chromatography and then digested with alkaline phosphatase as described in the Experimental Procedures section. The resultant nucleosides were then passed over a silica cartridge to separate ribonucleosides from deoxynucleosides. The ribonucleoside fractions were concentrated by evaporation and then chromatographed on the cation-exchange resin.



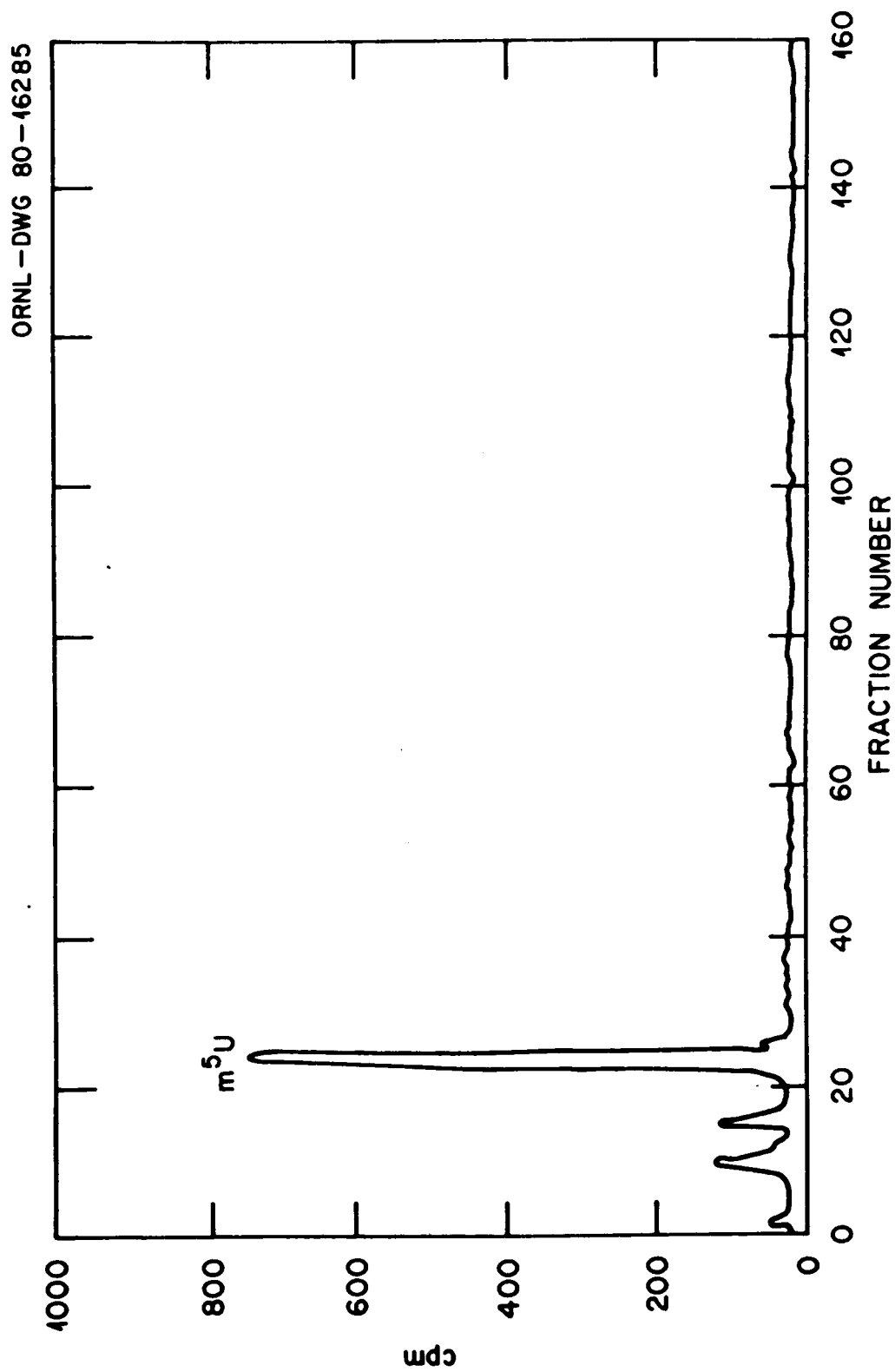


Figure 20

Figure 21. [ $^3\text{H-CH}_3$ ]-methionine labeled deoxynucleosides from alkaline phosphatase digestion of acid-soluble nucleotides. The soluble pools were prepared, digested and chromatographed on the cation-exchange resin as in the legend to Figure 20, except that the deoxynucleoside group resulting from alkaline phosphatase digestion of the nucleotides was chromatographed on the cation-exchange resin.

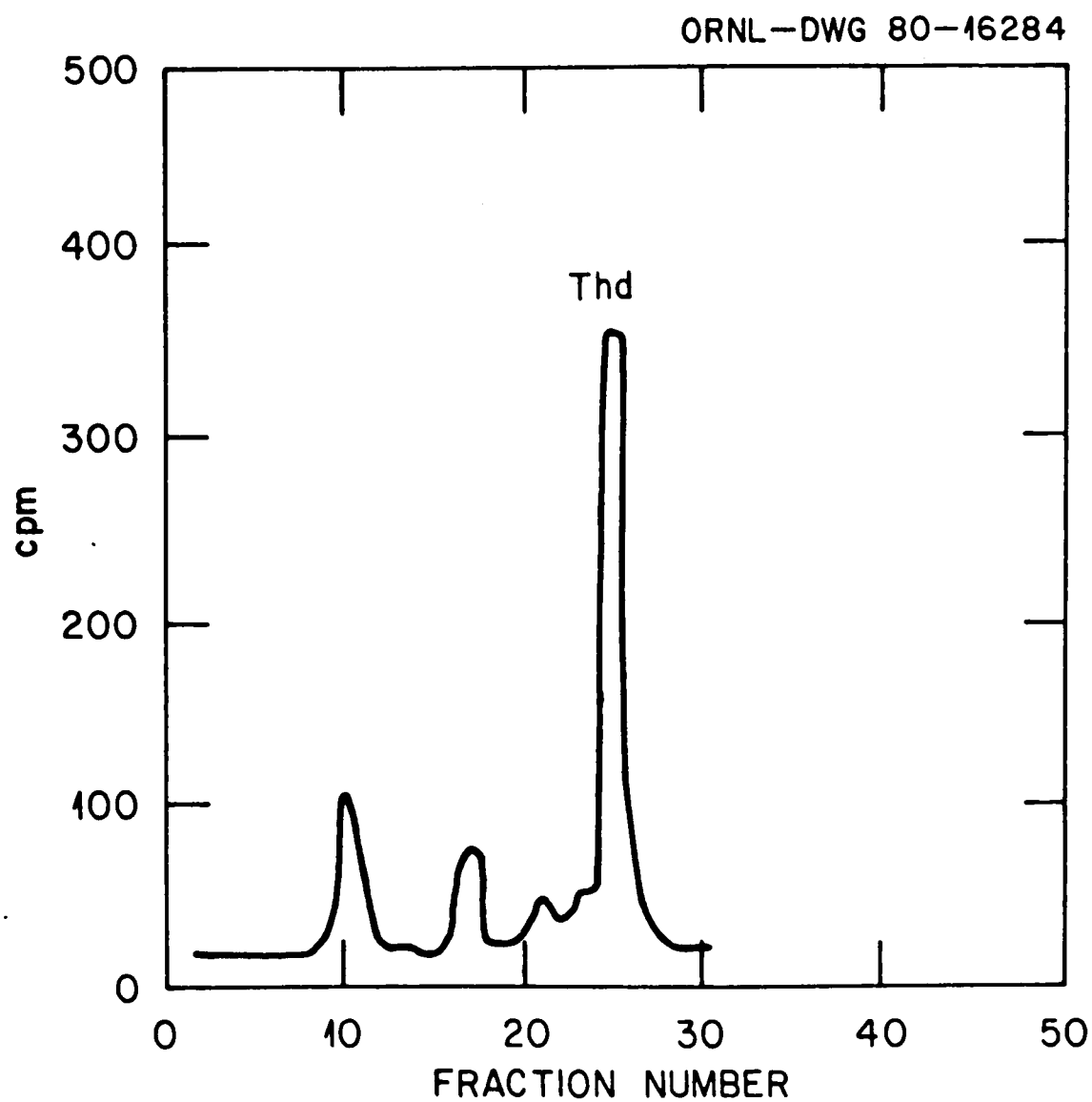


Figure 21

cells, media, or both. This observation suggests the labeling of thymine in DNA results from salvage of ribothymidine from RNA.

## VITA

Clinton D. Lothrop Jr. was born in Knoxville, Tennessee on January 9, 1956. He attended Bearden Elementary and Junior High Schools in that city and was graduated from Bearden High School in June 1973. The following September he entered Cleveland State Community College in Cleveland, Tennessee and was awarded the Associate of Science degree in June 1975. He then attended The University of Tennessee, Knoxville and was awarded the Bachelor of Arts degree in Zoology with highest honors in June 1977.

He then entered The University of Tennessee, Knoxville Graduate School of Biomedical Sciences in Oak Ridge, Tennessee in September 1977. He was awarded both a Hilton A. Smith fellowship from The University of Tennessee, Knoxville as well as a fellowship from the National Institute of Health. He received the Doctor of Philosophy degree in Biomedical Sciences in March 1981.

The author is a member of the Phi Kappa Phi honor society and the Genetics Society of America. The author, to date, has published papers in the journals of Analytical Biochemistry and Clinical Chemistry. The author is currently a student studying veterinary medicine at The University of Tennessee, Knoxville.