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MOLECULAR EVENTS BASIC TO CELLULAR RADIATION RESPONSE

by

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ABSTRACT

Our work during the past year has been focused on three areas related to the cellular effects of radiation. We have been exploring radiation effects on RNA, the regulation of gene expression and amino acid-nucleic acid interactions.

We have continued our studies on the radiation response of RNA in growing and confluent cells. We have derived radiation survival curves and demonstrated repair of potentially lethal damage in 3T3 cells. Studies of giant cell formation and turnover of ribosomal RNA in irradiated cells has demonstrated differences in growing and confluent cells.

We have sought evidence consistent with our hypothesis for regulation of eukaryotic gene expression with segments of RNA reutilized to prime new RNA synthesis. Data derived from the turnover of ribosomal RNA and the methylation pattern of ribosomal RNA during turnover are consistent with the possibility that a segment of 18s ribosomal RNA is being conserved during new RNA synthesis. We were unable to show reutilization of the 5' trinucleotide of 18s and 28s ribosomal RNA but did find a ribonuclease resistant oligonucleotide in 18s RNA which appeared to be reutilized.

In our studies of amino acid nucleic-acid interactions using nuclear magnetic resonance spectroscopy we have been able to successfully synthesize an amidate and begin an examination of the intramolecular interactions. We have also studied intermolecular interactions between tryptophan and nucleoside monophosphates and found upfield shifts which provide evidence for preferential stacking of the 6-membered ring of tryptophan with adenine and evidence for specific geometry of interactions of tryptophan with cytosine.
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I. Introduction

The effects of x-irradiation on whole animals and their multicellular tissues are manifold and complex. To better understand these effects much of modern radiobiology has been concerned with understanding radiation effects at the less complex cellular and subcellular levels. A clearer knowledge of the mechanisms of action at these more fundamental levels will help to explain the diverse effects seen at the multicellular level. Studies of cellular and subcellular radiation effects have at least in part been motivated by the desire and the necessity to reduce patient radiation effect in diagnostic radiology and nuclear medicine and increase the tumoricidal effect in therapeutic radiation and medicine.

Significant doses of x-irradiation at the cellular level lead ultimately to either malignant transformation or cell death. In both cases x-irradiation usually produces these effects by acting upon the sequence of mechanisms responsible for cell division. Clearly then we must understand how normal cells initiate and control their division process in order to discover how normal cells change in these regards with x-irradiation and neoplasia.

A. Laboratory Historical Background

Efforts to understand the biochemical mechanisms of cell division were begun in this laboratory in 1964. At that time we undertook a study on the mitotic apparatus of the dividing sea urchin egg. We derived optical rotatory dispersion data for the dissolved apparatus and found that these
data were close to those for the muscle protein actin. This suggested the possibility of an active contractile process for chromosome separation (1).

Several lines of evidence (2) suggested that the mitotic apparatus was not synthesized de novo at mitosis but was assembled from subunits formed earlier in the cell cycle, presumably in the $G_2$ phase. Moreover a very large proportion of the total cell protein at mitosis was thought to be mitotic apparatus protein. We therefore compared the pattern of protein synthesis during $G_2$ with the cell cycle as a whole in an attempt to differentiate one predominant band of protein synthesized during $G_2$ which might represent the mitotic apparatus protein. Proteins labeled with one isotopic precursor ($H^3$ leucine) during $G_2$ were mixed with proteins labeled with another isotopic precursor ($C^{14}$ leucine) during the entire cell cycle and separated on polyacrylamide gels. We compared the $H^3/C^{14}$ ratio down the length of the gels to find one band showing a marked increase that could be attributed to mitotic apparatus protein. Instead of one unique band we found a very complex array of differences in the pattern of protein synthesis in $G_2$ as compared to the rest of the cell cycle (3,4). Changes in patterns of protein synthesis form the biochemical basis for differentiation and are presumed to represent changing patterns of gene transcription. Our results describing changes in the pattern of protein synthesis during the cell cycle suggest that not only during development but also during a single cell cycle gene transcription may show variations in activation and repression.

These changes in the pattern of gene expression leading up to cell division are apparently initiated in the $G_1$ phase of the cell cycle, prior
to the DNA synthetic period. Once cells commit themselves to DNA synthesis they almost always go on to divide (5). For this reason our efforts focused on cells which ordinarily remain in a resting state (G₁ or G₀) until stimulated to divide. By understanding the mechanisms underlying this stimulation we hoped to gain some background to the problem of initiation of cell division.

Untransformed cells grown in tissue culture multiply until they cover their growth surface (become confluent), whereupon their mitotic rate either drops to zero or decreases markedly. At this time the cells are said to show "contact inhibition" (6,7). When more growth area is made available by trypsinizing and reseeding into new larger tissue culture flasks the cells once again start dividing rapidly until they again become confluent. At confluence the cells in the "monolayer culture" are in the G₁ phase of the cell cycle (8), as are most resting mammalian cells (5). Whether or not the in vivo condition is duplicated in tissue culture, contact inhibition does represent an entry into understanding cellular mechanisms that lead to cell division.

Investigations of intracellular changes following release of contact inhibition had been conducted on cells released from contact inhibition by reseeding cells in a larger growth area after trypsinization, by adding fresh serum (9,10) or by transforming these cells (11-13). These methods of releasing contact inhibition possess some disadvantages for the interpretation of experimental results. It is uncertain, for example, that early
biochemical changes seen after reseeding trypsinized cells are due solely to loss of contact inhibition. Some may represent changes necessary to repair cellular damage caused by the trypsinization.

Serum stimulation of division in contact inhibited cells does not cause physical cell separation, and biochemical alterations seen after addition of fresh serum to a confluent culture might be due to stimuli other than those operating in an authentic release of contact inhibition. Similarly, virus infection of contact inhibited cells can and does activate processes other than those simply concerned with cell-cell contact.

A method of releasing contact inhibition without disturbing cell substrate attachment, or the chemical composition of the medium, should provide for an increase in the space available for growth of a confluent culture. Ideally, such an increase in growth area should be uniformly available to every cell within the culture. Increasing the growth area along a border of the culture leads to stimulation of cell division in only a very small and insignificant fraction of cells immediately adjacent to the border. Cells within the center of the culture are not released from contact inhibition (14).

We developed (15) methods for releasing contact inhibition of division by increasing the area of growth of a confluent culture in a fairly uniform manner throughout the culture. Cells are seeded into culture dishes in the presence of small glass beads. The cells multiply between the beads until confluent. Contact inhibition is removed by discarding the beads, leaving behind numerous spaces in the culture, between the cells. The cells multiply
to fill these spaces until they are again confluent. Using this method we were able to describe the changes in protein, and RNA synthesis that occur after the release of contact inhibition. We were also able to show by comparison of two different mouse fibroblast lines that apparently not all resting cells are blocked in the same location within the G1 phase (16).

Elucidation of the earliest events following the release of contact inhibition requires knowledge of how cells in confluence signal their neighbors to stop dividing, and how they interrupt that signal when the cells have their growth area increased and are no longer confluent. We hoped that an understanding of this process of cell to cell communication would lead to an approach to unraveling the intracellular control mechanisms responsible for switching on the processes of cell division in resting cells, and for the alteration of such mechanisms in malignant cells.

We wished first to develop a method for studying the problem of transfer of macromolecules between cells. The method had to allow for biochemical examination of the transferred macromolecules. The desired method would also have to be able to recognize transfer between genetically and phenotypically similar cells.

A method (17) was developed to culture two populations of cells together in tissue culture and then separate the two populations. One population was then examined for labeled macromolecules which had passed over from the other. One group of cells was grown in the presence of tantalum particles (2 μ avg. diam.). These tantalum-containing heavy cells were added to fresh cells and the two groups of cells cultured together. After 5-20 hours, during which time the tantalum-containing cells and fresh cells became
confluent (i.e. in intimate contact with one another), the culture was trypsinized and the cells put on 0-17% ficoll gradients and centrifuged. The heavy tantalum-containing cells passed to the bottom while the light cells floated upon the ficoll. The light cells were removed and the labeled macromolecules transferred from the heavy donor cells were examined.

Using this method we were able to demonstrate transfer of RNA between cells (18). By several different methods we established that high molecular weight RNA was transferred but not precursors or degradation products. Moreover trivial explanations of our results such as presence of donor cells or donor cell fragments with the recipient cell fraction were ruled out. However no unique RNA species that could be related to maintenance of the contact inhibited state could be identified.

This same method also showed transfer of RNA between transformed cells (19), and intercellular protein transfer with transfer into cell nuclei (20). Interestingly, of all the nuclear protein fractions the histones showed the greatest degree of transfer.

Because of this finding of a relatively greater transfer of histones, we investigated the effects of histones on growing and confluent cell cultures. Some of the major histone fractions were found to increase cell number at confluence and to cause disruption of the normal ordered appearance of cultures at confluence (21). It is not clear whether this was an intracellular effect or was due merely to alterations caused in the cell membranes.

At this point in our work it seemed to us that the problem of cell division and its initiation was bound up with the general biological problem of regulation of gene expression. For if cell division is initiated early in
the cell cycle and if the cell cycle is accompanied by and presumably caused in gene expression, then the initiating trigger for cell division is probably found in the activation of one or more specific genes.

Although bacterial systems have mechanisms for gene control which have been well documented, those for eukaryotic cells appear to be more complicated. Such cells are probably controlled by different or additional genetic regulatory mechanisms to those described for prokaryotes. Because of the enormous number of genes to be controlled within a eukaryotic cell it would seem that only RNA or protein would have the necessary specificity to control specific gene transcription. RNA can use its well-documented property of hybridization to uniquely determine its interaction with genomic DNA. The mechanisms of specific protein and nucleic acid interaction are not known. We have therefore been studying by nuclear magnetic resonance methods interactions between amino acids and nucleosides in an effort to learn more about the specificity that these interactions possess. Knowledge of these interactions should help in understanding and predicting the possible macromolecular specificity between a gene and its repressor protein.

Because of the ability of RNA to hybridize with DNA we have investigated the possible role of RNA as an inhibitor of gene transcription. If RNA were acting to repress gene transcription then the more highly differentiated a cell, the more repressor RNA it would have. We have therefore examined a fully differentiated cell, which shows no genetic transcription, the chick erythrocyte, for the presence of DNA-associated RNA. Using in vitro labeling procedures, we found less than one ribonucleotide in association with $10^5$
deoxyribonucleotides of the avian erythrocyte DNA, which is far too little to possibly repress all the gene within a fully mature avian erythrocyte (22).

Although segments of RNA may not act as genetic repressors it is still possible that such segments could exercise genetic control as activators of transcription as proposed in several hypotheses (23-25). However none of these hypotheses propose a source for their activator RNA. We have therefore put forward a new hypothesis for gene regulation by activator segments of RNA which does postulate a source for this RNA (26,26). We propose that segments of RNA derived from breakdown of high molecular weight RNA act as primer for RNA synthesis by forming the 5' ends of new RNA molecules.

In studies on growing and confluent 3T3 cells (28,29) we found that ribosomal RNA does not show appreciable turnover in growing cells but does show turnover when the cells become confluent. This is consistent with other studies on chick fibroblasts (140). We found a single component decay curve if the cells were labeled after confluence was attained, similar to the results of
others (152). However, if the confluent cells were prelabeled when subconfluent the decay curve had two components. We have also examined the methylation pattern of 18s and 28s rRNA. These studies also showed that in confluent cells 18s rRNA was not turning over as rapidly as the 28s rRNA and that variations in the ratio of base to ribose methylation occurred during the turnover of 18s RNA but not 28s RNA. These results are consistent with the above model, assuming that 18s RNA lies at the 5' end of the 45s precursor molecule. When cells are in a steady state condition, merely turning over their ribosomal RNA, the 5' end of the ribosomal precursor is reutilized and is therefore not labeled. Turnover of label therefore involves only the nonconserved portion of the precursor. If, however, the cells are labeled while growing, there will be turnover at confluence of two labeled components; the short lived nonconserved RNA and the longer lived primer RNA.

During the past year we have been investigating the turnover of ribosomal RNA seeking direct evidence for the reutilization of the 5' end of either the 18s or 28s RNA in the synthesis of new RNA. We have labeled one group of subconfluent 3T3 cells with P\(^{32}\) and then chased the label during a 3 week period after confluence was attained and the cells were turning over their RNA. These cells were then combined with subconfluent cells freshly labeled with P\(^{33}\). The RNA was extracted and digested to oligonucleotide fragments with T\(_4\) ribonuclease. The digest was then treated with a spleen 5' exonuclease which was expected to digest all of the oligonucleotide fractions except for the 5' oligonucleotide which would be resistant to digestion because of its possession of a 5' phosphate group. The resulting
digests were then put on urea DEAE cellulose columns to isolate the resistant 5' oligonucleotide and to compare its ratio of $P^{32}/P^{33}$ to the rest of the molecule. We first ascertained the expected position in the column eluate of the 5' oligonucleotide by in vitro labeling of the 5' ends of 18s and 28s RNA with phosphokinase and ATP$^{32}$. This was found to be a trinucleotide.

On examination of the dual labeled eluate, the ratio $P^{32}/P^{33}$ of the 5' oligonucleotide fraction was identical to the major mononucleotide peak in both 18s and 28s RNA. However a much larger O.D. peak was found at an oligonucleotide length of about 15-20 nucleotides in both the 18s and 28s RNA digests. In 18s RNA this peak had a $P^{32}/P^{33}$ ratio which was 20-30% higher than the rest of the 18s molecule (the mononucleotide peak). However in 28s RNA the ratio of $P^{32}/P^{33}$ in this oligonucleotide peak was identical to the rest of the molecule. Since the turnover of 28s RNA is greater than 18s RNA, the $P^{32}/P^{33}$ ratio of 18s RNA as a whole is greater than 28s RNA. Therefore the even higher ratio in this 18s RNA oligonucleotide fraction rules out the trivial possibility that it is merely a contaminant from the 28s RNA.

The ribonuclease resistant oligonucleotides in both 18s and 28s RNA are labile to alkali suggesting that they are probably RNA and not a DNA contaminant.

The increased $P^{32}/P^{33}$ ratio of the 18s oligonucleotide suggests that it may be reutilized during turnover and for this reason we are now actively trying to further characterize it. We are now examining it for the presence of methylation. We are also trying to determine whether it may be a fragment of...
poly A containing messenger RNA that cosediments on sucrose gradients with 18s RNA. Its possible dual stranded nature will be under investigation.

In further studies on the 5' end oligonucleotides of 18s and 28s RNA, we have devised a thin layer chromatography procedure to separate the mononucleotide monophosphates from the 3'5' mononucleotide diphosphates of the form pXp which are present as the 5' end nucleotide of both 18s and 28s RNA. In both 18s and 28s RNA this 5' end is adenosine (pAp).

Other studies this past year have been initiated in the area of radiation effects on confluent and growing cells and differences in giant cell formation and repair of potentially lethal damage in confluent and growing cells.

By studying amino acid nucleic acid interactions with nuclear magnetic resonance spectroscopy we will gain some insight into protein-nucleic acid interactions which occur in gene regulation in prokaryotes and possibly eukaryotes as well. During the past year we have successfully synthesized amidates and examined intramolecular interactions in these molecules.

Interactions between tryptophan and adenosine monophosphates, cytidine monophosphate, adenylyl-3' → 5'-adenosine, adenylyl-3' → 5'-cytidine, cytidylyl-3' → 5'-adenosine and cytidylyl-3' → 5'-cytidine in solutions at low pH have been studied via protonmagnetic resonance spectroscopy. Upfield shifts of ring protons for all species have been noted as the ratio of tryptophan to mononucleoside phosphate was increased at constant total concentrations. Upfield shift magnitudes have been interpreted as evidence for preferential stacking of the 6-membered ring of tryptophan with that of the adenine residues, and as evidence for a geometry of interaction for tryptophan with cytosine residues such that the proton at position 5 on the cytosine ring is closer to the area of greatest ring current magnetic anisotropy of tryptophan.
5. Scientific Scope

Outline

I. Regulation of Gene Expression
   A.) Primer RNA
       1) Research Plan
   B.) Conservation of RNA Segments
       1) Ribonuclease resistant oligonucleotide
           a) Research Plan
   C.) Analysis of the 5' oligonucleotide of 18s and 28s Ribosomal RNA
       1) 5' oligonucleotide from exhaustive T1 digest
           a) Research Plan
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           a) Research Plan

II. Radiation Effects on RNA
   A.) Radiation Response of RNA in Growing and Confluent Cells
       1) Radiation survival curves for 3T3
       2) Repair of potentially lethal damage
       3) Giant cell formation
       4) Turnover of ribosomal RNA in irradiated cells
   B.) Research Plan
       1) Radiation induced alterations in transfer RNA
           a) Radiation induced RNA strand breakage
           b) Radiation induced alterations in the pattern of modified nucleosides
III. Amino Acid-Nucleic Acid Interactions

A.) Synthesis of amidates

B.) Intramolecular interactions of amidates
   1) Research Plan

C.) Intermolecular interactions between tryptophan and dinucleoside phosphates
   1) Research plan
II. Reports

A. Regulation of Gene Expression

Although some of the principles and mechanisms operating in the control of gene expression for inducible enzyme systems in microorganisms are now generally understood (30-32), the mechanisms for regulating gene expression in eukaryotes have not yet been fully elucidated. Bacterial control systems thus far studied are controlled by external agents and conditions such that simple changes in environment readily repress or derepress transcription. Bell (33) has pointed out, however, that eukaryotic enzymes are apparently all constitutive. Substrate induction and the methods of regulating the genetic process concerned with substrate induction may therefore be limited to microorganisms. There is no clear understanding of how gene control in eukaryotic cells is achieved during differentiation or during the cell cycle (34). Several features of gene regulation in differentiated cells tend to suggest that mechanisms additional to those seen in bacterial systems of enzymatic adaptation (30) are being utilized in these cells. Among these features are the large increase in mass of DNA in the eukaryotic cell, the nuclear processing of transcribed RNA to the smaller molecular weight cytoplasmic forms of RNA, the presence of repetitive DNA base sequences within the genome, and the programmed schedule of the steps of differentiation. In eukaryotes, once a cell is differentiated and mature it generally remains in such a state even when there are very gross changes in the environment (e.g. the persistence of in vivo characteristics on in vitro cultivation). Only upon the special situations represented by oncogenesis or regeneration is there limited reversion of particular less differentiated functions. The absence of ready reversibility of differentiated functions suggests that such functions may not be explicable by the well documented bacterial control systems utilizing substrate induction.
THEORIES OF EUKARYOTIC GENE REGULATION

Several theories (35-38) have been put forward to explain genetic control in eukaryotic cells. However, in none of these is there a suggestion of the source of the macromolecules required to interact initially with the genomic DNA nor an understanding of how their uniqueness and relation to the product RNA arise. Although RNA, because of its potential to base pair uniquely with DNA, plays a prominent role in these theories, the initial interaction with DNA that activates gene transcription, as distinguished from the secondary products, is postulated to be not with RNA but with some molecule other than RNA interacting with the DNA. Only after this initial interaction with DNA is there a stimulus for the production of activator RNA (36) or is a polymerase able to pass a site on the DNA on which transcription is restricted. These hypotheses do not specify how these initial interactions with DNA, which must be unique and specific for a great number of genes, take place. Therefore one must assume that in these theories the initiating interactions in complex differentiable eukaryotic systems are similar to those seen in inducible and repressible bacterial enzyme control systems in which protein repressors react directly with DNA (39,40).

Georgiev (33) suggests that RNA transcribed from eukaryotic DNA consists of two parts. The first part, on the 5' end, is non-informative, and is destined to be degraded within the nucleus. The second part is the informative part which is destined to become messenger RNA. The two parts together, that is the initial whole molecule, he proposes, is the heavy heterogeneous nuclear RNA reported by several laboratories (41-43). Various regulatory proteins interact with sites on the DNA from which the non-informative part of the RNA is transcribed. These regulatory proteins will determine whether or not RNA polymerase
can interact and the process of transcription can pass these sites.

Britten and Davidson (36) have presented another hypothesis for gene regulation in differentiated cells. Their theory is based on an analysis of system components needed for response of a cell to external signals. They suggest that inducing agents, probably bound to specific protein molecules, bind to sensor gene areas of DNA. This binding in turn causes the production of activator RNA sequences on integrator genes. The activator RNA produced then interacts with a receptor gene which is linked to a producer gene on which the actual messenger RNA is transcribed.

Crick (44) has postulated that if recognition sites on double stranded helical DNA are to be recognized in such models then the DNA will have to be unwound in the recognition region. Such focal unwinding would be stabilized by histone or non-histone nuclear proteins. Paul (37) has suggested that a destabilizing polyanionic molecule such as a non-histone protein causes a reduction in DNA supercoiling, allowing an RNA polymerase molecule to bind in the area of unwinding. However, the polymerase would have to pass regulator sites containing repressors or derepressors before it could proceed to transcribe the structural gene. The passage of the polymerase would be accompanied by RNA synthesis which would lead to further DNA unwinding due to the polyanionic character of RNA and thus further transcription of the structural gene.

Frenster (38) has suggested that DNA is kept in a repressed state by histones. These histones can be displaced by nuclear polyanions allowing a specific derepressor RNA to hybridize with single stranded DNA which would free the complementary strand of DNA for synthesis of messenger RNA. To regulate transcription of that particular gene Frenster proposes that the newly
synthesized messenger RNA would compete with the DNA for the complementary derepressor RNA, thus causing the DNA to return to its original repressed state, shutting off further messenger RNA production.

In none of the above theoretical considerations of the mechanisms controlling genetic regulation in differentiated cells is there a suggestion of the source of the macromolecules required to initially interact with DNA nor an understanding of how their uniqueness and relation to the product RNA arises.

MOLECULES INTERACTING WITH DNA

Control of gene expression in eukaryotes would seem to depend ultimately on the form of the primary chemical interaction with the genomic DNA. The interacting molecules would probably have to be of macromolecular dimensions in order to provide sufficient variations to interact specifically and uniquely with all of the genes in a mammalian cell nucleus. Lipids and polysaccharides do not have sufficient variations in their structure or sequence for this task. For this reason the aforementioned schemes for genetic control have postulated either proteins or RNA alone or in combination as the molecules which directly interact with DNA.

Nuclear proteins

The proteins described in association with DNA consist of RNA polymerases, histones and non-histone proteins. Studies have appeared on RNA polymerase from eukaryotic cells and have been recently reviewed (45). Three
polymerases have been described in mammalian cells. These polymerases have, however, no preference for a particular DNA template in an in vitro system (46). In amphibian embryos (47) no correlation was found between the synthesis of different classes of RNA and the amount or synthesis of polymerase I or II. All embryonic stages, both those making and those not making rRNA, contain both polymerases. In addition, both enzymes are present in amucleolate cells which do not make rRNA. These findings together with the limited number of polymerases suggest that specificity in gene transcription does not lie in unique RNA polymerases. Moreover there does not appear to be any profound stimulation of transcription with polymerase initiation factors from bacteria such as the sigma or rho factors.

Because of the striking similarity in amino acid composition and electrophoretic mobility of each of the major histone fractions from sources as widely disparate as pea seedling and calf thymus (48,49) and other considerations of the constancy in amount and proportion in varying cell conditions and cell types (50-59), it has generally been believed that histones do not have the necessary specificity to repress and derepress specific areas of DNA of higher organisms that would be necessary in such complex processes as cell differentiation. Although side chain modifications of histones (60) may confer some general complexity to histone interaction with DNA, as yet no detailed specificity has been assigned to these alterations in histone molecules.

Although various reports have appeared on nuclear acidic proteins affecting gene expression (61-67) some doubt has been cast on the validity of such studies in view of the great difficulty in isolating true chromatin preparations free of contaminating cytoplasmic proteins (68).
RNA interactions

If RNA were the macromolecular component involved with DNA in the control of transcription, then base pair interaction could provide a method for recognition of unique gene sequences. RNA could act either as a repressor of gene transcription or as an activator of RNA synthesis. If RNA acted as a repressor of gene transcription, then one would expect to find a maximal amount of repressor RNA in association with DNA in a fully repressed genome. However, on examination of the fully repressed DNA of the mature avian erythrocyte we found (69) insufficient DNA-associated RNA which could act as repressor sequences for all of the genome, even assuming repetitive genes could be controlled by one RNA repressor sequence. The mature, fully repressed avian erythrocyte nucleus was found to have less than one RNA nucleotide in association with 100,000 nucleotides of DNA. If RNA interactions with DNA are responsible for regulation of gene expression it seems unlikely that such RNA would be in the form of repressor molecules.

On the other hand, if RNA acts as an activator of transcription, as postulated in theories mentioned earlier, then a source for this RNA is necessary. If short RNA sequences serve as activators of RNA transcription, there are several possibilities for the origin of this activator RNA. It may be synthesized with or without the presence of a second activator RNA for the synthesis of this activator RNA. If its synthesis is activated by a second activator RNA, then from where arises the activator RNA for the synthesis of the second activator RNA and how does the chain of activator RNAs activating other activator RNAs begin? If the activator RNA is synthesized de novo then we are back to no hypothesis at all for the explanation of specific RNA transcription.
PROPOSED HYPOTHESIS

The following hypothesis (70,71) for the control of genetic transcription which might be operative in at least some cases of regulation of eukaryotic transcription. This hypothesis suggests that the initiating event for RNA transcription is the interaction of activator RNA with the genomic DNA and, unlike other theories, postulates a source for this RNA. After base pair interaction with DNA, the activator RNA then acts to prime the transcription of an RNA and becomes incorporated itself into the newly transcribed RNA.

RNA destined for the cytoplasm is transcribed, processed, if necessary in the nucleus, and then appears in the cytoplasm. After fulfilling its function, the cytoplasmic RNA is degraded. I propose that at least some of the degradation products of the cytoplasmic RNA or the processing procedures in the nucleus are the source of the primer RNA required by the hypothesis. High molecular weight RNAs would thus have component sequences within them which could, by hybridization, identify unique genes and cause the initiation of transcription of those genes. Segments of RNA, therefore, would be conserved after nuclear or cytoplasmic breakdown and utilized as initiating segments in the production of new RNA molecules.

The primer RNA would interact by base pairing with unique single stranded regions of DNA. Such single stranded control regions probably do exist, at least in bacterial DNA, as evidenced by the finding that the lac operator region in E. coli is hypersensitive to single strand specific nucleases (72). Single strand regions could be produced by a mechanism such as that proposed by Crick (44).
Primer RNA production and steady state conditions

Processing of high molecular weight RNA in the nucleus could produce the primer RNA required by the hypothesis. If heterodisperse nuclear RNA were to degrade its 5' end in the nucleus, as suggested by the experiments of Georgiev et al (73), and the initial portion of that 5' end fragment were conserved to act as a primer RNA, then that RNA 5' end could base pair with the same 3' end of the DNA gene site from which the transcription of the degraded RNA was initiated. Synthesis of a new RNA identical to the degraded molecule could thus be primed. The new molecule would possess at its 5' end the conserved initial 5' fragment of the degraded RNA molecule. This process is illustrated in the figure by scheme I, steps 1a, 2a, and 3.

Degradation of RNA molecules in the cytoplasm following the completion of their function might produce conserved fragments which could move back into the nucleus and by base pair recognition act as primers for the synthesis of identical RNA molecules. This process is illustrated in the figure by scheme I, steps 1b, 2b, and 3. The breakdown, for example, of a particular messenger RNA (mRNA) within the cytoplasm would produce primer RNA, which could initiate the synthesis of an identical daughter mRNA molecule to replace the degraded parent molecule. Fragments from old RNA molecules which act as initiators of identical new RNA molecules could be used to maintain a constant number of any particular RNA molecule during the steady state conditions of a differentiated cells. If only the 5' fragment is conserved, then only one identical molecule will be reproduced and the amount of that particular RNA would remain constant. Other similar feedback control systems could be imagined which would amplify the supply of a particular RNA by having not only the 5' end fragment of the molecule conserved, but also an internal
sequence of the molecule which repeats that 5' sequence conserved, resulting in more than one primer RNA.

**Primer RNA production and developmental sequences**

If some segments from internal regions of an RNA molecule are conserved after degradation, these segments might hybridize to the 3' ends of genes different from those used for transcription of the degraded RNA. These segments from internal regions of degraded molecules could then prime the transcription of RNA different from the degraded RNA, with the internal segment of the degraded molecule appearing at the 5' end of the newly transcribed RNA. This process could lead to a developmental sequence, as follows. The breakdown of a mRNA could result in the production of one or several primer segments derived from internal regions of the mRNA molecule. These segments could then prime one or several other mRNA's different from the original mRNA molecule. These latter molecules, in turn, on breakdown, could lead to production of other primers and other messenger RNAs. In this manner, a developmental sequence could be constructed, characterized by a programmed series of differential gene transcription. This is illustrated as scheme II of the figure.

The developmental scheme would require that some primer sequences of the DNA genome are repeated in the internal segments of other DNA gene sites. To assure that the RNA primer fragments did not select the internal gene regions from which they were initially transcribed to prime new RNA transcription, it would be necessary that transcription be primed only at the 3' end of gene sites. Mechanisms such as local unwinding (44) or the single stranded nature of such 3' end sites (72) could be envisaged to restrict the sites of hybridization of RNA primer segments.
The original source of macromolecular RNA for animal cells would then be maternal mRNA stored in the ovum (74). The breakdown of this mRNA in the post fertilization period would lead to activator RNA priming further messenger production. After many cycles of synthesis of RNA determined by primer segments from degraded RNA and breakdown of this RNA leading to further RNA synthesis, a fully differentiated cell is obtained. A fully differentiated non-metabolizing cell (e.g. erythrocyte) might contain no mRNA because the final step in the developmental process would be to activate the synthesis of an RNA coding for a ribonuclease which would completely degrade any previously synthesized RNAs. Other metabolizing but fully differentiated cells would be left in a steady state balance where breakdown of mRNAs led to primer RNAs for the same message.

Mitosis would be expected to partition cytoplasmic and nuclear RNAs between the two daughter cells. Thus, the particular RNAs and their conserved segments inherited by each cell would determine the particular developmental pathway chosen by that cell.

Such an hypothesis could explain not only the programmed schedule of differentiation but also the persistence of phenotypical characteristics of certain differentiated cells for long periods of time under considerable environmental change and stress. Such cells might not exhibit phenotypical changes simply because they do not have the primer RNAs to initiate these changes. If changes do occur (other than through genetic selection processes dependent on conditions favoring selection of cells with mutant DNA), then they either come about through the possible intervention of foreign primer RNAs or chance alterations in ribonuclease specificity. The foreign primer RNAs could come from embryonic interactions (75,76), from other cell to cell
transfer of RNAs (77), from introduction of a viral genome or from RNA found in
the extracellular matrix (78,79).

Consideration of the size of polyoma DNA and virus specific proteins made
by an infected host suggests that the polyoma viral genome does not have
sufficient nucleotides to code for all the viral specific proteins made by
the infected cell (80). Perhaps breakdown of the RNA produced on the viral
genome provides sufficient primer RNAs to synthesize all the required viral
specific mRNAs.

Elimination of primer RNA

Reutilization of degradation fragments as primer RNA segments could lead
to additional controls on the development of differentiated functions. The
breakdown of an RNA molecule could lead to the production of primer segments
not only for a mRNA involved in the next step of differentiation, but also, if
developmentally necessary, for mRNAs that will code for a ribonuclease specific
for complete destruction of a particular messenger or its primer sequence to
mono- or small chain oligonucleotides. The production of such ribonucleases
could provide a further feedback control system during steady state conditions.

The above hypothesis requires a mechanism for eventually degrading the
primer sequences when the particular RNA is no longer required. Very little
is known about the mechanisms and kinetics of RNA degradation and the specificity
of the ribonucleases required. It is possible that the resistance to destruction
of primer segments is only a relative resistance in comparison with the rest
of the molecule and that after several cycles of priming RNA transcription,
the primer itself will succumb to destruction. Alternatively, as the program of
development proceeds, an RNA segment might be conserved which would prime for the transcription of a mRNA for a class specific ribonuclease which would be capable of destroying both the primer RNA's which preceded that particular stage of development, as well as its own mRNA.

POSSIBLE SUPPORTING EVIDENCE FOR HYPOTHESIS

Several lines of evidence in support of this theory might be predicted. 1) One would expect to find that RNA synthesis requires the presence of activator or primer RNA; 2) there might be evidence of RNA transfer from cytoplasm to nucleus (although nuclear RNA processing could lead to activator RNA sequences whose entire lifetime would be intranuclear); 3) a class of low molecular weight metabolically stable activator RNA should be found intracellularly; 4) there should be evidence that intracellular breakdown of RNA need not be complete to mononucleotides; and 5) direct examination of cytoplasmic RNA should show evidence of conservation of RNA segments. That is, the 5' end of newly synthesized RNA molecules should consist of a reutilized fragment of RNA.

Activator RNA

Some features of the process of DNA transcription on the DNA template of bacterial cells are now known (81-83). This synthesis requires an RNA polymerase and several initiation factors which have been isolated and used in in vitro studies. RNA oligonucleotides may act to stimulate transcription by priming RNA synthesis. Niyogi and Stevens (84), studying E. coli RNA polymerase reactions using synthetic polyribonucleotides as templates for
synthesis of complementary polyribonucleotides, found that complementary synthetic oligonucleotides stimulated the action of RNA polymerase. They also provided strong evidence for these complementary oligonucleotides acting as chain initiators for the RNA polymerase reaction. Other studies (85,86) of transcription of bacteriophage have shown that, while in the presence of nucleotide triphosphate precursors, almost no DNA transcription takes place whereas dinucleotides dramatically stimulate RNA synthesis, possibly by acting as primers.

Studies (87) of eukaryotic RNA polymerase have also shown only a low level of RNA synthesis with native DNA as template because of a failure to initiate RNA chains. It may be that activator RNA absent in these in vitro studies and acting as the initial sequences in the RNA to be transcribed provide the necessary initiation for the RNA chains. Investigations of eukaryotic polymerase referred to in a previous section have shown that RNA synthesis is not initiated on synthetic polynucleotide templates (88). Perhaps activator RNA associated with extracted DNA is necessary to initiate transcription. Kanehisa, et al (89) have shown that chromatin low molecular weight RNA from chick liver had specific stimulatory effects on RNA polymerase when chick liver chromatin was used as template but inhibitory activity when E. coli DNA was used as template. Perhaps activator RNA specific for chick was present amongst the chromatin RNA.

Frenster (90) has added various nuclear polyanions including nuclear RNA to isolated repressed chromatin and has measured RNA synthesis. The greatest increase (more than twice that of the other fractions) in RNA synthesis was found with added RNA. Brown and Coffey (91) have also shown that polyribonucleotides can enter rat liver nuclei and there affect genetic transcription.
RNA transfer from cytoplasm to nucleus

Goldstein and his coworkers (92,93) have shown that in amoeba there is a class of small molecular weight RNA that moves from the cytoplasm to the nucleus. These workers, after labeling amoeba nuclear RNA, transplant the labeled nucleus to another amoeba and then observe small molecular weight labeled RNA within the host nucleus. They have also done successive nuclear transplants with the host nucleus transplanted into a new cytoplasm followed by transplantation of the new host nucleus. They have also transplanted an unlabeled nucleus into an amoeba containing labeled cytoplasmic RNA and observed labeled RNA in the transplanted nucleus. Their work suggests that there is a class of small molecular weight nuclear RNA that is synthesized in the nucleus, (possibly as part of larger molecules) that enters the cytoplasm and then returns to the nucleus. By radioautographic analysis they have demonstrated that some of this RNA is associated with the chromatin (94). These RNAs may represent, if our hypothesis were correct, the activator RNA sequences arising from degraded cytoplasmic RNA that return to the nucleus to initiate new RNA transcription.

Other evidence for the ability of cytoplasmic factors to activate genetic transcription has been described. Harris (95) found that when the fully differentiated avian erythrocyte with a completely repressed genome was transplanted into a HeLa cell, the nucleus began to swell and to synthesize RNA. Possibly, the cytoplasmic factors involved were RNA molecules activating transcription in the transplanted nucleus. Other studies, utilizing transplantation of nuclei from one species or one stage of development to another, have shown the decisive influence of cytoplasmic factors
on differentiation in development. These studies have been reviewed in amoeba (96), amphibia (97), and insects (98). It is certainly possible that the cytoplasmic factors involved are activator RNA segments.

**Low molecular weight RNA**

The presence and stability of a class of low molecular weight RNA which could function as primer RNA is required by the proposed hypothesis. Low molecular weight RNA has been found in the nucleus (99-102) in the extracellular matrix (79) and in the medium of cells cultured in vitro (78). A rather constant property of these classes of low molecular weight RNA is their metabolic stability.

**Resistance to complete breakdown**

The stability of the low molecular weight cellular RNA and its resistance to ribonuclease degradation would be of importance if such RNA acted as primer in the proposed hypothesis. The nuclear low molecular weight RNA, for example, is only slowly labeled with such precursors as $^{32}\text{P}(101,103)$. The stability of some of the low molecular weight RNA that has been reported can probably be linked to the extensive methylation of these species of RNA. Methylation has been shown to confer ribonuclease resistance on RNA (104-107). Methylation may provide one means, therefore, of regulating the extent of RNA breakdown and the production of well defined sequences of primer RNA.

Although there is evidence for limited methylation of mRNA (109), a similar function of regulating the extent of RNA breakdown could also be provided by double stranded regions of mRNA (109-113). These regions have been shown to be resistant to ribonuclease (114,115). Jelinek and Darnell (114)
estimate that the ribonuclease resistant double stranded regions of high molecular weight nuclear RNA comprise about 3% of these molecules. Ryskov et al (116) found ribonuclease stable RNA sequences in the high molecular weight nuclear RNA which are removed during the nuclear processing of this RNA to the size of mRNA. The stability of these sequences is due to double stranded regions, probably in hair-pin like structure. Perhaps such stable products of RNA processing in the nucleus perform the role of primer RNA.

Patnaik and Taylor (117) have also described low molecular weight, ribonuclease stable, nuclear RNA. This RNA is polydisperse, possibly part of the heterodisperse large nuclear RNA, and appears to be not entirely base paired except at certain regions which may represent the necks of loops. Such low molecular weight RNA could be part of the breakdown products of larger molecules which because of their stability in the presence of RNAase could be conserved to provide the primer RNA required by my hypothesis.

**Conservation of RNA segments**

Cheevers and Sheinin (118), from kinetic experiments on the incorporation of labeled uridine into polyribosome-associated mRNA, found that a portion of this RNA turned over with a lifetime of about 2 hours but that another portion had a much longer lifetime, greater than 6 hours. Singer and Penman (119) measured directly the rate of degradation of H\(^3\) uridine labeled polyadenylic acid containing mRNA. They found a two component decline in radioactivity of mRNA with one component having a half-life of 6-7 hours and the second showing a 24-hour half-life. These results on mRNA are consistent with the hypothesis that the two components represent different segments of RNA molecules and that the longer lived components represent segments reutilized as primer RNA.
Ribosomal RNA does not turn over in growing cells but does turn over in confluent cells. Studies of our own (120) on ribosomal RNA turnover in confluent cells in culture have shown a single component decay curve if the cells are labeled after confluence is attained, similar to the results of others (121). However, if the confluent cells are prelabeled when subconfluent, the decay curve has two components. These results are consistent with the above model. When cells are in a steady state condition, merely turning over their ribosomal RNA, the 5' end of the ribosomal RNA precursor is reutilized and is therefore not labeled. Turnover of label therefore involves only the nonconserved portion of the precursor. If, however, the cells are labeled while growing, there will be turnover at confluence of two labeled components; the short lived nonconserved RNA and the longer lived primer RNA.

Several investigators (122-124) have found that even after relatively short pulses of radioactive precursors, radioactive RNA remains in the nucleus during chase periods of up to several days. Fakan and Bernhard (122) suggest that since the rapidly labeled heterodisperse nuclear RNA has a short lifetime, then this persistence of label even up to four days post-incubation represents reutilization of the products of metabolism of RNA. While it is possible that the persistence of this label represents reutilization of mononucleotides, it is also possible that whole sequences of nucleotides may be reutilized as required by the proposed hypothesis.

Repetitive DNA sequences have been described (125) in the eukaryotic genome. Such repetitive sequences are consistent with the proposed hypothesis. If internal segments of an RNA molecule were preserved on degradation to act as primer for new molecules then one would expect to find a DNA sequence within
the interior of a gene which repeats itself at the 3' end of another gene. This would also suggest that the 5' ends of RNA molecules should show hybridization to repetitious DNA to a greater extent than other portions of these molecules. Dina et al (126) have in fact shown that the 5' ends of mRNA from Xenopus embryos are transcribed from repetitious DNA sequences.

OTHER CONSIDERATIONS

This hypothesis does not preclude the existence of other mechanisms for regulating gene transcription. Several studies have demonstrated the possible role of hormones in the regulation of gene expression (127-129). Although such regulation undoubtedly plays a role during development and in the response of cells to environmental stimuli it would appear that such influences could be entirely responsible for the complicated sequential pattern of gene expression that occurs in a developing organism.

This hypothesis also does not exclude the possibility that other theories discussed earlier can be integrated with it. The hypothesis suggests an origin for the activator RNA which those other schemes require. This model also proposes a possible explanation for the programmed steps in developmental pathways. Since preparation of this manuscript and the first presentation of this hypothesis (70) suggestions similar to some of those made here, based on considerations of ribonuclease specificity, were discussed by H.D. Robertson and E. Dickson at the 1974 Brookhaven Symposium (130).

One implication of this hypothesis is that the integrity and fidelity of RNA and its sequence is of equal importance to that of the genomic DNA in pathways of development and the maintenance of the differentiated state in
eukaryotic cells. In a sense, therefore, genetic inheritance also resides in RNA. Changes or mistakes in RNA sequence in segments of a molecule destined to become primer RNA could lead either to failure of hybridization of the primer or, if the change were great enough, hybridization to an incorrect gene site. This would result either in absence of synthesis of a particular RNA molecule or synthesis of an incorrect molecule.

In general, the validation of this hypothesis will depend on experiments involved in RNA sequence determination. Study of sequences at the 5' end of newly synthesized molecules should show, for example, by radioactive labeling techniques, that these are of older age than the remainder of the molecule: In addition there should be persistence of labeled segments from one molecule in the 5' region of new molecules. Studies are now under way to study these possibilities.
1.) Turnover of Ribosomal RNA

During the past two years we have been studying the kinetics of turnover of ribosomal RNA to ascertain whether segments of RNA were being conserved.

Ribosomal 18s and 28s RNA are derived from a single 45s precursor molecule (131). Several studies (132,133,139) suggest that the 18s molecule lies at the 5' end of the 45s precursor. Other work (134, 135) suggests that the 28s molecule lies at the 5' end of the 45s precursor. We have therefore studied the decay of radioactivity in the 18s and 28s RNA when these molecules are turning over to find evidence for conservation of radioactivity in either 18s or 28s RNA not found in the other ribosomal RNA species.

We have studied the stability of $^3$H uridine labeled ribosomal RNA in growing and confluent cells (136). Subconfluent cultures were radioactively labeled with $^3$H uridine and then chased with a large excess of unlabeled uridine while still subconfluent and growing. The chase was continued during the next two weeks while the cultures grew to confluence and were maintained in a nongrowing confluent state. At intervals during the chase period radioactivity was determined in 18s and 28s RNA isolated from sucrose gradients of RNA extracted with phenol from duplicate cultures. After an initial rise in radioactivity, which was probably due to failure to completely dilute out radioactivity in the uridine pool after the onset of chase (137,138), the radioactivity remained constant until confluence was attained, after which radioactivity began to decline. This lack of turnover during growth, and the presence of turnover at confluence of ribosomal RNA, was consistent with the findings of others (139-141) that ribosomal RNA does not turn over during growth but does turn over in confluent contact inhibited cells.
Hemocytometer cell counts at confluence during the chase period showed no significant decline in cell number and there were no mitotic cells seen after confluence was attained. $^1$C thymidine radioactivity administered and chased on the same to duplicate $^3$H uridine labeled cultures also showed no significant decline during growth or confluency. These findings would indicate that the turnover of $^3$H uridine radioactivity was due to RNA turnover rather than cell loss.

The kinetics of degradation of ribosomal RNA were measured by incubating growing subconfluent cell cultures with $^3$H uridine and chasing the cells with an excess of unlabeled precursor after confluence had been reached. Radioactivity in 18s and 28s RNA was measured at intervals during a three week chase period. The decay curve of radioactivity in labeled 18s RNA showed at least two components. The first component had a half life of about 2.5 days and the second component had a half life of about 6 days. The decay curve for uridine-labeled 28s RNA, on the other hand, was linear on a semi-logarithmic plot, indicating a single component. The half life of about 2.8 days for the decay of 28s RNA was close to the half life of the first component of the 18s decay curve.

Similar two component decay curves for 18s RNA and single component decay curves for 28s RNA were seen in cells which had been labeled with $^3$H methyl methionine and chased when confluent for three weeks.

The variation between 18s and 28s RNA decay curves seen in the case of RNA labeled while growing suggested that ribosomal RNAs might possess some degree of
heterogeneity in regards to susceptibility to degradation. Since changes occur in RNA metabolic activity (139-142) at confluence, it seemed worthwhile to also investigate the decay of radioactivity in ribosomal RNA species in cultures labeled while confluent, rather than labeled while in logarithmic growth as in the preceding experiments.

Confluent cultures were radioactively labeled and chased for three weeks in a manner similar to the subconfluent cultures described above. Radioactivity in both 18s and 28s RNA from cultures labeled while confluent with H\textsuperscript{3} uridine showed single component decay curves during a three week chase period. For 18s the half life appeared to be about four days. Radioactivity in 28s appeared to decline with a half life of about 3.5 days. Similar one component decay curves were found for 18s and 28s labeled in confluent cultures with H\textsuperscript{3} methionine and chased for three weeks.

The half life of unstable ribosomal RNA has been calculated in other systems to follow first order kinetics and to be between 12 and 121 hours (139,140, 143-146). In our experiments, except for the case of the 18s RNA prelabeled in growing cells which will be further discussed, we also found single order kinetics with a half life of unstable ribosomal RNA of 2 1/2 to 4 days. This half life is intermediate in the range found by others. Differences in half life could possibly be explained by differences in the cell types used and differences in the generation times of the cell types. It is interesting that Murphy and Attardi (147) found a half life of 3 days for messenger RNA labeled for 24 hours in growing HeLa cells, which is close to the half life of rRNA of our results in cells labeled for 24-48 hours. Other investigators (148,149) have, however, found a shorter half life for messenger RNA after labeling for shorter periods.
The 18s RNA prelabeled in growing cells shows second order kinetics in its decay. The first component is similar in lifetime to that of the 28s RNA and to the lifetime of 18s RNA prelabeled in confluent cultures. The second component appears to have a half life of about 7 days. This second component could represent a contaminating species of RNA that sediments with 18s RNA on a sucrose gradient or a form of 18s RNA that is more resistant to degradation. Nair and Knight (150) have shown that intermediates in the degradation of 28s RNA can sediment with 18s RNA. However it is unlikely that the second component in the decay curve of 18s RNA, labeled while growing, represents a degradation product of 28s RNA, because it does not appear in the decay curve for 18s labeled while confluent. It has been shown (142) that during confluence there is a change in the ratio of membrane-bound to free ribosomes. Perhaps such differences could account for the differences in stability of 18s RNA depending upon the location of the prelabeled RNA.

Several investigators (148,149, 151) have shown a two component decay curve for poly A-associated mRNA in exponentially growing cells that do not show contact inhibition. In the light of our results with 18s RNA it would be of great interest to examine the kinetics of turnover of mRNA in contact inhibited cells prelabeled while growing and prelabeled while confluent.

A report (152) has appeared which demonstrates differences in the rates of turnover of 18s and 28s rRNA in resting 3T3 cells. Confluent cells were labeled with radioactive precursors and the cells were chased for 10 days. They found, as we did with cells labeled when confluent, single component decay curves for 18s and 28s rRNA. Their data did not include cultures labeled while growing and chased when confluent as in our experiments.
These results are consistent with our hypothesis for the regulation of gene expression. We have suggested that RNA transcription may be regulated by the presence of primer RNA. These primer RNA's are segments from the normal breakdown of functional cytoplasmic or heterodisperse nuclear RNA. If a segment of RNA from the 5' end of a degraded RNA molecule is preserved, then it can hybridize with the 5' portion of the gene from which it was originally transcribed and thereby act as a primer to resynthesize and replace the entire degraded RNA molecule. Such a scheme might explain steady state turnover of ribosomal RNA in confluent cells. Ribosomal RNA is synthesized from a 45s precursor molecule.

It is not certain whether the 18s rRNA or the 28s rRNA is at the 5' end of the 45s precursor. Although some studies have suggested that the 28s rRNA is near the 5' end of the 45s precursor (153,154) other studies (150,155,156) indicate that the 18s rRNA is at the 5' end of the precursor while other studies (158,159) tend to refute the evidence on which electronmicroscopy was used to show that the 28s rRNA is at the 5' end of the precursor. If the 18s portion of the molecule is at the 5' end of the precursor while the 28s portion is at or near the 3' end of the precursor, a conserved 5' primer segment might be expected to be within the 18s molecule. In the decay of 18s RNA labeled while growing, the 5' end is conserved and therefore shows a longer half life than the remainder of the molecule when turnover begins at confluence. If however, an 18s RNA molecule is labeled while confluent, the 5' end being conserved will not be labeled. Therefore only the nonconserved labeled portion of the molecule will turn over and only a one component decay curve will be seen. The 28s RNA, being at the 3' end of the 45s precursor, would show
only a single component decay curve since none of this molecule is conserved to be used in the synthesis of new RNA molecules. This would be the case whether it was derived from cells labeled while growing or labeled while confluent.

It is of interest that an endonucleolytic enzyme has been described which attacks 28s RNA at multiple sites. The same enzyme has a more restricted attack on 18s RNA to yield a species more resistant to endonucleolytic digestion (159). Perhaps in vivo that more resistant portion of the 18s molecule is conserved to initiate new ribosomal RNA transcription.
2.) Comparison of Turnover Kinetics of 18s vs 28s RNA

The hypothesis that we have proposed for the regulation of gene expression predicts that segments of RNA from the degradation of RNA are reutilized in the synthesis of new RNA molecules. Such segments act as primer for new RNA synthesis and therefore appear at the 5' ends of newly synthesized molecules. If the 5' end of the 45s ribosomal precursor is 18s RNA, then, as turnover occurs, the 5' end of the 18s molecule is conserved. If ribosomal RNA is labeled while growing, and then chased while confluent, therefore, the ratio of radioactivity in 18s vs 28s RNA should increase since 28s is completely degraded while a portion of 18s is conserved during turnover. We have therefore labeled 18s and 28s RNA during culture growth and observed the radioactivity in each species during a chase at confluence. We have also examined radioactivity in ribosomal RNA during a chase from cells labeled while confluent when the conserved 5' segment would not be expected to be labeled and therefore, the ratio of activity between 18s and 28s would not be expected to increase since radioactivity in both 18s and 28s would be completely turning over.

Growing cell cultures were labeled with radioactive H3 uridine for 24 hours and then chased for three weeks in the presence of an excess of non-radioactive uridine. At intervals during the chase period the 18s and 28s ribosomal RNA peaks, separated on sucrose gradients, were analysed for radioactivity. Labeling within the major ribosomal RNA species was expressed as a function of the optical density. Such a function eliminated problems of different relative amounts of each species in each experiment. Such differences could arise from problems in phenol extraction, alcohol precipitation, non specific RNA degradation or non lineairities in sucrose
gradients. Optical density should be a measure of the amount of RNA present (128), and although each RNA species, because of its configuration, may differ in this relation, it is assumed that for each RNA species this relation is constant for each experiment. The ratio of radioactivity to optical density within each peak was determined ($r_{18}$ and $r_{28}$) and these ratios compared by another ration $R$ ($R = \frac{r_{18}}{r_{28}}$).

Our data showed no significant change in the $R$ values while the prelabeled cultures were growing. After confluence was attained, however, there was a rise in the $R$ value, suggesting greater label in the 18s RNA. Cultures were also labeled while confluent and sacrificed without a chase. In that case the $R$ value was significantly lower than that seen with the cultures labeled while growing, suggesting less label in the 18s RNA than the 28s RNA in the cultures labeled when confluent in comparison with the cultures labeled while growing.

Growing cultures were also labeled with $H^3$ methionine and chased while confluent. Our data showed a rise in the $R$ value for the $H^3$ methionine prelabeled cultures during the chase, indicating, as in the $H^3$ uridine labeled experiments, a relative increase in the proportion of label within the 18s peak when compared to the 28s peak. Cultures labeled while confluent and not exposed to a chase also showed a smaller $R$ value when compared to the cells labeled while growing. These results with $H^3$ uridine and $H^3$ methyl methionine were consistent with our hypothesis that suggests reutilization of 5' segments of degraded RNA as primer at the 5' end of new RNA molecules.

Several studies (139-141,143) besides our own discussed above have demonstrated that in eukaryotic cells in culture, ribosomal RNA accumulates without turnover as long as the cells are growing. When the cultures become confluent and
growth ceases the cells begin to show ribosomal RNA turnover. Two laboratories (139,141) have reported increased radioactive label in 28s RNA as compared to 18s RNA at confluence after a short labeling period using relatively short chase periods. Since both 18s and 28s are derived from the same 45s precursor, and are presumably synthesized from the same nucleotide pools, these workers have postulated that there is either significant 18s RNA degradation shortly after its appearance, with a somewhat longer lifetime for newly synthesized 28s RNA (141) or that there is a lengthened transcription time of the 45s ribosomal RNA precursor at confluence (139).

Our results indicate that this asymmetry in label persists for a very long chase period and gradually increases during the chase. It would seem unlikely that either asymmetrical lifetime or change in transcription time could explain this asymmetry in 18s vs 28s label in long chase experiments suggesting that some other mechanism such as the one we have postulated involving conservation of RNA segments is responsible for the long chase asymmetry.
3.) Methylation Patterns of 18s and 28s RNA during Turnover

We have also examined the methylation patterns of 18s and 28s RNA during turnover. Since 18s RNA shows a two component decay curve in RNA labeled while growing, it was thought possible that these two components could have different patterns of methylation. Since methylation is known to restrict ribonuclease digestion it was thought that perhaps specific methylated nucleotides would cause resistance to degradation and therefore preservation of RNA for a primer function. The primer, with its specific labeled methylated nucleotides, would then appear in the 28s RNA. As the chase continued, the 18s RNA fraction would become enriched with those specific methylated nucleotides.

Base and ribose methylation were determined by paper chromatography by the methods of Tamaoki and Lane (160). This method separates mononucleosides from dinucleosides. Ribose methylated nucleosides appear in the dinucleoside fraction. Base methylated nucleosides were further analyzed and separated on thin layer chromatography by the methods of Randerath et. al. (161).

When $^3$H methyl methionine is used to label RNA some of the label appears in the intracellular C-1 pool and is used in purine synthesis. To limit this C-1 labeling of purines, sodium formate is added to the labeling medium. Even with sodium formate present in the labeling medium, however, some radioactivity does appear within purine rings and contributes to the radioactivity found within the mononucleoside spots on paper chromatograms. Since modified nucleosides account for only a very small percentage of total nucleosides almost all of the ring label appears in the major unmodified nucleosides.
Therefore to correct for ring label in the mononucleoside spots on paper chromatograms the percentage of total mononucleoside radioactivity that was in the methylated nucleosides found from thin layer chromatograms was multiplied by the total mononucleoside radioactivity found on the paper chromatograms to arrive at a correct value for methylated mononucleoside radioactivity. Although methylated nucleosides from RNA breakdown are not reutilized for new RNA synthesis, unmodified nucleosides, including purine ring labeled nucleosides, can be reutilized. Therefore during long chase periods the percentage of labeled mononucleoside in RNA that is attributable to purine ring labeling increases and the correction for ring labeling becomes more significant.

Using the above methods, no appreciable change was seen in the ratio of base methylation to ribose methylation during the entire three week chase period in either the 18s or 28s RNA. On comparison of maps of radioactivity on thin layer chromatograms of base methylated nucleosides over a three week chase period, no qualitative differences could be discerned. The ratios of activities among the various base methylated nucleosides remained roughly constant. The pattern of methylation of either 18s or 28s ribosomal RNA during turnover therefore did not change.

Brown and Attardi (162) have examined the methylation of rRNA in HeLa cells. By perchloric acid hydrolysis they were able to separate methyl radioactivity in the bases from the activity in the ribose. They found that 70-80% of the methyl groups were present on ribose. Using paper chromatography and paper electrophoresis to study acid hydrolyzed HeLa rRNA, Iwanami and Brown (163)
found a lower percentage of ribose methylation, about 40-50%. Our data, in 3T3 cells corrected for labeling of the purine ring, showed a higher percentage of ribose methylation in both 18s and 28s RNA. About 90% of the methyl groups in 18s RNA from 3T3 are located on the ribose. In 28s rRNA 93-94% of the methyl groups appear to be on the ribose. This higher degree of ribose methylation may be due to species difference between 3T3 and HeLa cells.

Since there was insufficient radioactivity for radioautography in the minor nucleosides separated on TLC, it is difficult to compare the base methylated components of HeLa and 3T3. However, a comparison of the in vitro labeled minor nucleoside trialcohol map of t-RNA derived by Randerath (161) with our maps of rRNA derived from 3T3 cells suggest that the most heavily labeled components in both 18s and 28s RNA are methyl-6-adenine, methyl-3-uridine and methyl-5-cytidine. Data on HeLa rRNA indicate that methyl-6-adenine, dimethyl-6-adenine and methyl-4-cytidine are the most common base-methylated minor nucleosides, suggesting further differences in the methylation of RNA in these two species. Since our method of labeling provided only a crude assessment of abundance of a particular minor nucleoside, it is not possible to be sure that small differences in the pattern of labeled methyl groups was not occurring during chase. On the other hand no major changes during the chase could be observed by inspection.

When a 45s precursor molecule is synthesized in the presence of radioactive methionine a certain small percentage, (even though 2 M sodium formate is used) of the label becomes incorporated into the purine ring and enters purine pools.
The ratio of ring label in 18s vs 28s RNA will depend upon the ratio of purines in 18s vs 28s RNA. Upon degradation of this RNA the labeled purines will reenter their respective pools and be used in the resynthesis of new 45s RNA. The resynthesized RNA should have the same ratio of labeled purine rings in 18s and 28s RNA as the degraded RNA. (Although because of pool dilution the total amount in each species will be reduced). Methyl labeling on the ribose or as methylated minor bases, on the other hand, occurs at the poly-nucleotide stage so that such methyl groups are not reutilized. Therefore as a chase continues the ratio (Q) of ring labeling in the major bases to total label increases in both 18s \( Q_{18} \) and 28s \( Q_{28} \) RNA.

Assuming that degradation is random, the ratio of \( Q_{28}/Q_{18} \) should remain constant because the resynthesized RNA has the same ratio of labeled purine rings in 28s vs 18s RNA as the undegraded 28s vs 18s RNA. However examination of our data showed a \( Q_{28}/Q_{18} \) ratio that increased with the chase period. Since our previously described work (136) had shown a two component decay curve for 18s RNA labeled while growing and chased while confluent, the current data suggested that the 18s RNA may be made up of two components, with the longer lived component showing fewer minor nucleosides. The overall pattern of nucleoside methylation, however, would have to be similar for the two components because of the similarity during the chase of the maps of activity on the thin layer chromatograms.

We can also explain this change in \( Q_{28}/Q_{18} \) by our previously discussed hypothesis that a segment of RNA from the 5' end of some RNA molecules is conserved on breakdown of the molecule and this 5' segment acts as primer for transcription of new RNA. In the case of 45s ribosomal RNA precursor, the
5' end may become 18s RNA (132,133,139). During turnover of ribosomal RNA, the 5' portion of the 18s RNA would be conserved. Since ring labeled nucleosides are reutilized while other methyl labeled nucleosides are not, this would cause a greater increase in the ring labeled to total methyl labeled nucleosides in 28s RNA which turns over completely ($Q_{28}$) than $Q_{18}$ which retains a conserved portion and does not turn over completely. $Q_{28}/Q_{18}$ would therefore increase as the length of chase increased, just as found in our data.
4). Primer RNA

Our previously discussed hypothesis for gene regulation in eukaryotic cells suggests that small molecular weight species of RNA derived from breakdown of high molecular weight RNA are used as primer to initiate new transcription. One prediction of this hypothesis is that RNA transcription might be accelerated in cells exposed to high concentrations of small molecular weight RNA derived from similar cells. To test this prediction, cellular RNA, extracted with phenol, could be added to cells in culture. In that case however considerable destruction of the exogenous RNA would occur because of nucleases in the medium. Moreover, the mononucleotide breakdown products of the exogenous RNA could enter the cell, alter the nucleotide pool sizes and in this way modify the rate of RNA transcription.

For these reasons we have investigated RNA transcription in an essentially cytoplasm free nuclear system. This system requires nucleoside triphosphates for transcription and therefore changes in the mononucleoside pools do not effect the rate of transcription. Moreover, there is no problem of cellular uptake of the exogenous RNA or its destruction by serum nucleases.

These cytoplasm free systems, so called "ghost monolayers", of nuclei (164) are emptied of their cytoplasm by the use of the detergent NP-40. Although their cell membranes are then freely permeable, their nuclear membranes remain intact and transcription is still active. Thus these ghost monolayers approximate the well known bacterial in vitro transcription systems. The uptake of radioactive uridine triphosphate into acid precipitable products can be measured during a ten minute pulse of radioactivity. Using such a
system in 3T6 cells we have been able to show a 41% increase in the uptake of 
$^{3}$H uridine triphosphate over a 10 minute period at 37°C if 5 optical density 
units per ml of whole cytoplasmic 3T6 RNA is added to the incubation mixture.

In recent studies using myeloma cell nuclei (165) Marzluff et al have 
shown that transcription can be maintained for longer periods in these 
nuclei if incubation is carried out at 25°C rather than 37°C. In 
addition they provide strong evidence that these nuclei are not only 
extending previously synthesized nucleotide chains but are also initiating 
new chains.

We have incubated ghost monolayers of 3T6 nuclei at 25°C and followed 
the incorporation of $^{3}$H uridine triphosphate into TCA precipitable products. 
At 37°C the incorporation levels off after about 15 minutes of incubation. 
At 25°C however the incorporation continues at about the same rate for 
longer than 80 minutes. This persistence of transcription for longer 
periods at 25°C than 37°C in 3T6 nuclei is similar to the above findings in 
myeloma nuclei.
5. Conservation of RNA Segments

In a previous section we have presented a new hypothesis for the regulation of gene expression in eukaryotic cells. One feature of this hypothesis is that degradation products of high molecular weight RNA are conserved to act as primer segments in the resynthesis of new high molecular weight RNA. When there is a steady state turnover of RNA the 5' end fragment of a particular RNA is conserved. It hybridizes to the 3' end of the DNA gene site of that particular RNA and primes the production of an identical RNA to replace the RNA that has been degraded. Thus a steady state amount of a particular RNA is maintained. To test this feature of the hypothesis we have been studying the synthesis and turnover of ribosomal RNA.

As previously shown in our work (136), as well as the work of others (139-141, 143), growing cells in culture do not turn over their ribosomal RNA. They begin to turn over their ribosomal RNA when they become confluent. If ribosomal RNA synthesized during turnover is primed by a conserved RNA segment, then examination of the 5' end of an RNA species should show reutilized fragments. If ribosomal RNA is radioactively prelabeled prior to confluence, when confluence is attained, and the RNA turns over, radioactivity in the 5' end of that RNA should remain and appear higher than the remainder of the molecule, since the 5' end is not turning over.

Whether 18s or 28s ribosomal RNA is located on the 5' end of the 45s ribosomal RNA precursor is not certain at this time. Several studies provide strong evidence that the 18s molecule is at the 5' end of the 45s precursor.
Recent electron microscope studies (153,154) suggests that
the 28s RNA lies at the 5' end of the 45s precursor. However other evidence
(157,158) suggests that this data may not be universally correct. We have
therefore studied the 5' ends of both 18s and 28s RNA and compared each to the
remainder of their respective molecules and to each other.

We have labeled the ribosomal RNA of growing cells with $^{32}$P and then
chased the label with fresh media without $^{32}$P for two weeks. These cells
were then combined with subconfluent cells labeled with $^{33}$P. The combined
cells were phenol extracted to yield RNA. 18s and 28s RNA fractions were
then obtained from sucrose gradients.

The 5' oligonucleotide segment of both 18s and 28s were obtained and
the ratio of $^{32}$/P$/^{33}$ determined in the 5' fragment and compared to this
ratio in the rest of the molecule. A $^{32}$P/$^{33}$P ratio in the 5' end segment
higher than in the rest of the molecule would indicate that the 5' end
segment was being conserved.

First the 18s and 28s ribosomal RNA molecules were broken down to
oligonucleotide fragments by complete T1 ribonuclease digestion and then an
attempt was made to digest all the fragments except for the 5' end fragments
with spleen exonuclease.

Spleen phosphodiesterase is an exonuclease specific for digestion from
the 5' end of RNA molecules (168). In order for this 5' exonuclease to digest
an RNA chain the oligonucleotide must end in a 5' hydroxyl group. If there
is a phosphate group on the 5' position the enzyme will be severely inhibited
(169). The oligonucleotide digestion products from T1 digestion contained
5' hydroxyl groups on the 5' end and cyclic 2', 3' phosphate groups on the 3'
end (170), with one exception. Since 18s and 28s RNA have a 5' phosphate group on the 5' end of these molecules then on hydrolysis to oligonucleotide fragments the oligonucleotide from the 5' ends possessed phosphate groups on the 5' end. If all the oligonucleotide fragments are exposed to spleen phosphodiesterase then there should be complete exonucleolytic digestion of all fragments except for the oligonucleotide from the 5' end of the original molecules, because that oligonucleotide has a 5' phosphate group which makes it resistant to enzymatic digestion. Ralph, Young and Khorana (171) have successfully used similar techniques for examination of the 5' end group of tRNA.

To separate the 5' oligonucleotide from the other products of spleen phosphodiesterase digestion the products of the enzymatic digestion were placed on DEAE cellulose columns (172). Radioactivity within fractions from the DEAE cellulose columns were determined and the $P^{32}/P^{33}$ ratio plotted for the entire column.

The fraction of the column containing the 5' end oligonucleotide was determined in the following manner. Unlabeled 18s and 28s RNA was prepared and the 5' end nucleotide then labeled with $P^{32}$-ATP, using phosphokinase (173). After T1 and spleen exonuclease digestion the RNA oligonucleotides were separated on DEAE cellulose columns. The radioactivity, which was associated with the 5' end oligonucleotide fragment, was predominantly located in a fraction that had a charge of - 6 in both 18s and 28s RNA and therefore contained a trinucleotide. The ratio of $P^{32}/P^{33}$ in this fraction was, in both 18s and 28s, identical to that in the large mononucleotide peaks which represented
the majority of each molecule. It would appear, therefore, that the
5' end oligonucleotide of neither 18s or 28s is conserved.

We have also compared the $^{32}\text{P}/^{33}\text{P}$ ratio in the 5' end nucleotide with
the ratio in the rest of the molecule. After alkaline hydrolysis of the
RNA dual labeled as above, the hydrolyzate was separated in two dimensions
on thin layer cellulose chromatograms. Because the 5' end nucleotide has
a phosphate group at the 5' position this nucleotide contains two phosphate
groups after alkaline hydrolysis. All other nucleotides contain only one
phosphate group except for the 3' end nucleotide which contains no phosphate.
By using 0.5 molar ammonium formate pH 7 in the first dimension and 2 molar
sodium formate pH 3.4 in the second dimension on thin layer chromatography
a satisfactory separation of mononucleoside monophosphates and mononucleoside
diphosphates was obtained. The radioactive label in the nucleoside di-
phosphates of both 18s and 28s RNA was concentrated at a position which
cochromatographed with 3'5' adenosine diphosphate, indicating that adenosine is
the 5' end nucleotide of both 18s and 28s RNA. After eluting this 5'
end nucleotide the radioactivity was compared to that eluted from the
mononucleotide spots. The ratio of $^{32}\text{P}/^{33}\text{P}$ in this 5' end mononucleotide in
both 18s and 28s RNA was identical to the rest of the molecule. Thus we
found no evidence for conservation of the 5' end nucleotide of either 18s or
28s RNA during ribosomal RNA turnover.
a) **Ribonuclease Resistant Oligonucleotide**

In the previous section are outlined experiments that have been done to study the turnover of the 5' end oligonucleotide of 18s and 28s RNA. Those experiments suggest that the 5' end oligonucleotide as isolated from DEAE cellulose columns is not conserved during ribosomal RNA turnover. The 18s and 28s RNA were digested with T₁ ribonuclease and spleen exonuclease and then chromatographed in urea on the DEAE cellulose. The eluates from the columns were characterized by an initial very high mononucleotide peak, then a much smaller dinucleotide peak, probably due to the presence of methylated ribose, and then no peaks until a low broad peak appeared at an oligonucleotide length of about 15-20 nucleotides.

In each of the DEAE cellulose column eluates thus far investigated for 18s and for 28s RNA this low optical density peak has been present. This peak therefore appears to represent a stretch of RNA that is resistant to digestion by both T₁ ribonuclease and spleen exonuclease. Surprisingly the ratio of P³²/P³³ in this peak in the eluates from 18s RNA were in every case 20-30% higher than the rest of the 18s molecule. In the case of the 28s RNA molecule however, this peak has always had the same P³²/P³³ ratio as the rest of the 28s molecule.

This difference in the P³²/P³³ ratio between the 18s RNA ribonuclease resistant oligonucleotide (RRO) and the rest of the 18s RNA molecule suggests that this oligonucleotide may represent a segment of RNA conserved during ribosomal RNA turnover. Several experiments have been done to further characterize this ribonuclease resistant oligonucleotide.
The 18s RRO is not a contaminant of the 28s RNA since the ratio of $^{32}\text{P}/^{33}\text{P}$ in the entire 18s RNA molecule is greater than that in the entire 28s RNA molecule and the ratio in the RRO is even higher than the 18s RNA molecule as a whole. The difference in the $^{32}\text{P}/^{33}\text{P}$ ratio between 18s RNA and 28s RNA as a whole is not surprising because, as pointed out earlier, we (136) and others as well (152) have found that 18s RNA has a longer lifetime than 28s RNA.

The RRO peak in both 18s RNA and 28s RNA is not present in the column eluates if the RNA is hydrolyzed with alkali before it is put on the column, indicating that it is not a contaminating piece of DNA.
3. Radiation Effects on RNA

There are several considerations that suggest that the integrity of intracellular RNA may be important for the genetic and phenotypic stability of eukaryotic cells. It has been shown that normal cells possess RNA dependent DNA polymerase (181). Therefore it is possible that RNA sequences could be transcribed into genetic DNA. Recent studies (182-184) also suggest that both virally directed and endogenously directed DNA synthesis may be primed by segments of RNA. Fidelity of the RNA may be important, therefore, to proper gene replication. The hypothesis that we have recently proposed, and which is discussed earlier in this document, suggests that segments of degraded RNA serve as primer for new RNA synthesis by the base pair recognition by these segments of the 3' ends of DNA to be transcribed. It is clear that if this hypothesis is correct then fidelity of these RNA segments would be crucial to transcription of proper genes.

There have been numerous studies on the effects of radiation on nucleic acids, principally DNA, and on DNA transcription (185). In aqueous solutions free radical formation from water molecules is thought to be the immediate result of irradiation, with subsequent interaction of these free radicals with the nucleic acid bases. Hydration products of uracil, cytosine and deoxycytidine (186,187) have been described as well as cyclobutane type dimers (188-190). These compounds appear to be the principal photoproducts formed by U.V. irradiation. Studies (191,192) have also been done on the photoproducts of purines as well as pyrimidines. These studies, done in
the presence of alcohols, demonstrate photoalkylation at the C-8 position (193). Other studies on the radiation of polynucleotides demonstrate DNA strand breakage, a decrease in the rate of DNA replication, and the formation of interstrand linkage (185,194).

Although there is extensive research on the biochemical and genetic effects of in vivo and in vitro irradiation of DNA, very little has been established about the biochemical effects of irradiation of RNA. The effect of radiation on the inactivation and photoreactivation of tobacco mosaic RNA (195,196) and bacteriophage (197) has been studied. In addition, the loss of amino acid acceptor activity in transfer RNA has been investigated (198,199), and evidence for the inactivation of \( \beta \) galactosidase messenger RNA in E. coli has been presented (200). Studies on whole cell irradiation with subsequent analysis of RNA synthetic patterns and composition are, as yet, very sparse. Studies employing various ionizing and particulate radiations have demonstrated specific alterations in macromolecular RNA metabolism dependent upon the quality of the radiation (201). It has also recently been shown that radiation induced interphase death of thymocytes can be directly correlated to specific radiation induced lesions in RNA (202).
1). **Radiation response of RNA in growing and confluent cells**

The major cellular species of RNA are the 4s transfer RNA and the 28s, 18s, and 5s ribosomal RNA. Messenger RNA makes up only a small percentage of the total cellular RNA. For this reason we have elected to study initially only the radiation response of ribosomal and transfer RNA. It has been shown by several studies (139,140) including our own (136) that ribosomal RNA does not turn over in growing cells but does turn over in confluent cells. Changes also occur in the rate of synthesis of RNA in confluent cells in comparison to growing cells. Because we wished to have a picture of the radiation response of RNA under a wide range of conditions, we elected to study the radiation response of RNA in 3T3 mouse embryo fibroblasts which show excellent density dependent contact inhibition of division (16). Moreover, this laboratory has had extensive experience with this particular cell line and the variations of RNA biochemistry in these cells under different culture conditions (16-22,28,29).

In any study involving the action of radiation on cells or cellular components it is necessary to define the basic radiation response of the cell line involved. This is the case for numerous other cell lines (203-205). The methods involved in such determinations in radiobiology are very well defined (206). In its general sense, response does not mean simply the cell survival at a given dose but is intended to include characteristics such as repair, morphological alterations, enzymatic changes and modification of normal activities (e.g. turnover of ribosomal RNA (28,29,204,205,207-209)).
a). Radiation Survival Curves for 3T3

Complete radiation survival curves have been obtained for 3T3 cells both in the absence and presence of feeder layers. In both cases the curves obtained were similar to those normally obtained for other cell lines with a $D_0$ (the dose required to reduce survival by 1/e on the logarithmic portion of the curve) of 153 rads and a value of $n$ (the extrapolation number) of 5.

These values differ greatly from those reported for 3T3 by Nadolney (210) who found a $D_0$ and $n$ of approximately 65 rads and 45 respectively. While no other reports on this cell line are available the values that we obtain are consistent with values reported for almost every other cultured mammalian cell line including V79 (211), CHO (212), L (213) and many more (214,215,216,217). The presence of feeder cells only served to increase the plating efficiency by a factor of 2 (approximately 35%-70%) with no alteration in the survival curve.

b). Repair of Potentially Lethal Damage

Several recent studies on the repair of potentially lethal damage (PLD) have been reported in numerous cell lines (204,205). It has been found that conditions which potentiate this repair include overlaying plateau phase cells with conditioned medium (218) or balanced salt solutions (205) as well as simply allowing the cells to remain in a resting, confluent state following radiation (204,205,218). Repair of PLD in exponentially growing cells has not been found except under certain growth conditions which are suboptimal, again
including conditioned medium (218) and other solutions which will not support growth (205). Variations in this repair with dose and cell line have also been reported (204, 205, 218, 219).

Our studies have shown that repair of potentially lethal damage occurred in 3T3 cells with an enhancement of survival by a factor of approximately 3.5 when confluent cells were left undisturbed for 12 hours in conditioned medium. For exponential phase cells no enhancement was found when the cells were overlaid with Dulbecco modified medium solution (+glucose) without serum for 12 hours.

The magnitude of repair of PLD in confluent cells observed here is lower than that obtained under similar conditions for most cell lines (204, 205, 218, 219). Some authors, however, have shown a total absence of repair under these conditions (220). The inability of 3T3 cells to repair in the absence of serum is unexpected since repair of PLD in most cell lines is enhanced under these conditions (213, 221, 222). Further studies involving repair of PLD in 3T3 cells under suboptimal conditions of growth are now in progress.
Phase cells reseed with fresh medium immediately following radiation.

Undisturbed confluent monolayers while the highest ( C > 90%) was in exponential growth (10-50%) the lowest percentage of giant cells ( C < 10%) was found in Ptd (228). The same conditions which were found to enhance repair of growth (206-208) the same conditions which were noted under conditions not conducive to which promoted cell growth (10-20% medium exponential growth phase Ph). Gc formation was known to be dose dependent (208) cell line dependent (207,208) and dependent upon when the observation of size were made (206,208).

It has also been shown that giant cell formation is restricted predominantly to established, cell lines (206,208) although radiation induced giant cell formation has been noted in vitro (207). Giant cell formation is restricted predominantly to established cells lines (206,208). It has also been shown that giant cell formation is restricted to cells in culture and is a common histological finding in tissue after irradiation in humans (207) although radiation induced giant cell formation has been noted in vitro (207).

In studies of irradiated 3T3 cells we found a correlation between the results for repair of Ptd and those of giant cell formation. Giant cells were defined as cells having an area at least twice that of control cells under the same growth conditions. Giant cell formation predominated in irradiated populations which were maintained in conditions which were conducive to growth. Less giant cell formation was noted under conditions not conducive to growth (10-50%), which were found to enhance repair of Ptd (228).

Giant cell formation is known to be dose dependent (208) cell line dependent (207,208) and dependent upon when the observation of size were made (206,208). Giant cell formation is known to be dose dependent (208), cell line dependent (207,208) and dependent upon when the observation of size were made (206,208). Giant cell formation is known to be dose dependent (208), cell line dependent (207,208) and dependent upon when the observation of size were made (206,208). Giant cell formation is known to be
d). Turnover of Ribosomal RNA in Irradiated Cells

Our previous studies (28,29) have shown that in unirradiated populations, growing 3T3 cells do not turn over their rRNA while confluent cells do so in a characteristic manner. Following x-irradiation it is found that both groups of cells, growing and confluent, turn over approximately 70% of their rRNA within 16 days with the vast majority of this turnover occurring in the first 6-8 days (223). Since the response in both cell groups is essentially the same it is reasonable to assume that a common mechanism is involved. Also, since the kinetics involved in the post radiation turnover differ greatly from either of the preradiation controls, it is also reasonable to assume that the mechanisms of, or the reasons for, this new mode of turnover are quite different.
C. Amino acid-nucleic acid interactions

An understanding of the molecular basis of interactions between proteins and nucleic acids is fundamental to the elucidation of the mechanism of gene repression in prokaryotes. Although little is known about the nature of gene repression and activation in the more complicated genetic regulatory systems in eukaryotes it is possible that proteins, by interacting with specific regions of DNA, also regulate gene expression in eukaryotes. For this reason we have undertaken investigations on the interactions between amino acids and nucleic acids in an effort to uncover a basis for the specificity in the possible macromolecular protein-DNA interactions. Such work should also prove of value in understanding other protein nucleic interactions such as between an aminoacyl transfer RNA ligase and its cognate tRNA.

It has long been recognized that electrostatic interactions play an important role in the binding of basic polypeptides to nucleic acids. Electrostatic interactions cannot explain specificity, however, as the negatively charged phosphate groups are evenly distributed along the entire length of the nucleic acid chain. More recently, investigations have focused on the possible roles of hydrogen-bonding and hydrophobic forces. Of the hydrophobic-type interactions which may be involved in complex formation, ring-stacking between the pyrimidine and purine bases of the nucleic acid and the aromatic side chains of the protein's amino acid residues (phenylalanine, tryosine, tryptophan, histidine) seems to be a particularly viable possibility. A number of studies have been made using a variety of model systems - aromatic amino acids and dipeptides with nucleosides and dinucleoside phosphates (233); aromatic amines (234,235).
aromatic amino acids (236,237), or peptides and peptide amides containing aromatic residues (238-240) with DNA any polynucleotides; tryptophan and indole derivatives with the purine and pyrimidine bases, nucleosides and nucleotides (241). Interactions between the components of these model systems have been investigated by luminescence (fluorescence and phosphorescence) and reflectance studies, nuclear magnetic resonance (NMR) spectroscopy, ultraviolet (UV) absorbance spectroscopy, circular dichroism (CD), optical rotatory dispersion (ORD), equilibrium dialysis, and measurements of viscosity, sedimentation rates and melting temperatures. Almost all of the results point to the same interpretation: namely that the aromatic amino acids form complexes with the nucleic acids or their component nucleotides with ring-stacking interactions between the residue side chains and the nitrogenous bases; the aromatic rings of the amino acids being partially or wholly intercalated between the stacked bases in the case of double or single stranded DNA or of polynucleotides (such as polyadenylic acid).

Perhaps the most informative source of data has been nuclear magnetic resonance spectroscopy; it provides a particularly convenient technique for the study of complex formation between aromatic molecules in solution because from NMR spectra it is possible to detect both hydrogen-bonding and ring-stacking interactions and to readily distinguish between them. If hydrogen-bonded complexes are formed, the expected response is a down-field shift of the proton resonances of both molecules. On the other hand, stacking interactions between the aromatic rings will tend to induce upfield shifts of the aromatic proton resonances in both molecules due to the magnetic anisotropy (ring current) effects of each molecule on the other (237). If the exchange is fast between free and complexed molecules
on the NMR time scale - the expected case for a weak complex - only one resonance peak will be observed for each individual proton in the equilibrium mixture. The observed frequency will be a weighted average of the resonance frequencies in the free and bound states (242). In addition to this information about the nature of the interaction in the complex, it is also theoretically possible to determine the stoichiometry, association constants, thermodynamic parameters (enthalpy and entropy), and the stereochemistry of the complex from the concentration and temperature dependence of the magnitude and direction (with respect to the applied magnetic field) of the changes in chemical shifts for the different protons of one of the molecules (243).

It is possible to analyze the NMR data according to simple mathematical models which take into account both the possibility of self-association and of complex formation (of several stoichiometries) through ring-stacking interactions (243).

Experimental studies on aqueous solutions of aromatic amines or amino acids with nucleosides or nucleotides clearly indicate that complex formation leads to upfield shifts of the proton magnetic resonances associated with the aromatic ring protons of both components (233, 237, 241, 244, 245). The NMR spectra, however, show no line broadening, indicating that the exchange rates between components in the complex and free molecules are very high; the chemical shift of a given proton, then is obtained as an average over free and complexed molecules (245). Helene et al. (244) have found that the ribose protons of the nucleosides or nucleotides and the protons of the amino acid chain are also displaced upfield upon complexation,
but to a much smaller extent, the largest displacement being observed for $H_1$, which is closest to the purine or pyrimidine ring. A decrease in the coupling constant between the $H_1$ and $H_2'$ protons of the ribose (244,245) might indicate that the ribose conformation is changed during the interaction between the nucleoside or nucleotide and an aromatic amino acid; it is possible, judging from the change in the coupling constant, that the conformational change represents a change of the ribose puckering from 2'-endo in the free nucleotide to 3'-endo in the complex (245).

The experimental evidence above has been interpreted as being indicative of a ring-stacking interaction between the aromatic moieties of the component amino acids (amines) and nucleosides (nucleotides), the upfield shifts of the protons being caused by the effects of the ring current magnetic anisotropy of each aromatic ring on the other molecule. Comparison of the relative magnitudes of the upfield shifts for different protons has led to the conclusion that the six-membered rings rather than the five-membered rings of the purine bases and of the indole system of tryptophan and tryptamine (and other indole derivatives) are preferentially involved in ring-stacking (233). The observed chemical shifts also indicate that the aromatic rings are stacked parallel to each other in the complex (241).

Calculation of association constants, $K$, from the changes in chemical shift reveals that complex formation is markedly more extensive when it involves a purine rather than a pyrimidine base. Wagner and Lawaczeck (245) have determined that the contribution of the nucleotide base to complex stability...
decreases in order: guanine > adenine > thymine > uracil > cytosine.

Association constants for complexes involving tryptophan are considerably larger than the corresponding complexes with tyrosine and phenylalanine (237). In any case, association constants are relatively small and range from 1 M\(^{-1}\) for the binding of tyramine with cytidine (pH 7.5, 25°C) (237) to 9 M\(^{-1}\) for a tryptamine-5'GMP complex (pD 7.9, 37°C) (245).

The largest upfield shifts of the heterocyclic base protons in such complexes are induced by tryptophan and related indole derivatives. This is readily explained by the differences in intermolecular shielding values due to the ring current magnetic anisotropies of the aromatic amino acid side chains: Giessner-Prettre and Pullman (246) have calculated that shielding decreases in the order tryptophan > phenylalanine > tyrosine histidine, with tryptophan exhibiting considerably larger shielding values than any of the others. Among the nucleotide bases, adenine induces the largest upfield shifts in the aromatic amino acid residues. The intermolecular shielding due to ring current magnetic anisotropy of the bases has been calculated (again by Giessner-Prettre and Pullman) (247) to decrease in the order: adenine > guanine > cytosine > uracil. Thus, complex formation between tryptophan (tryptamine) and adenosine is the case most easily studied by NMR spectroscopy, as the interaction between these two molecules gives rise to the largest upfield shifts of aromatic protons on both components. The extrapolated upfield chemical shifts for the aromatic protons of tryptamine complexed with 5'-AMP (at 37°C and pD 7.9) are in the range of 0.3 ppm (245).
NMR studies of complexes formed between aromatic amines, amino acids or peptides containing aromatic residues and nucleic acids also reveal upfield shifts in the resonance peaks of the aromatic protons of the amino acid side chains; on the other hand, no observable difference between the NMR spectra of a free peptide and of the corresponding DNA-peptide complex is noted when the peptide does not contain an aromatic amino acid (248). Moreover, the resonance peaks of these aromatic protons are markedly broadened in the DNA and polyA complexes whereas those of the aliphatic protons are not. This broadening is interpreted as reflecting a greater restriction of mobility of the amino acid aromatic ring in the complex as compared to other parts of the oligopeptide (239). As noted previously, it is not possible to obtain high-resolution NMR spectra of double-stranded DNA, so that changes in the chemical shifts of the base protons cannot be followed upon complex formation. However, in complexes of aromatic amino acids and peptides with polyadenylic acid, a downfield shift of the \( H_2 \) and \( H_8 \) resonance peaks of adenine have been observed (236,239). This indicates that the disruption of adenine-adenine interactions due to complex formation causes a stronger downfield shift than can be balanced by the ring current effects of the aromatic amino acid side chains on the bases which would tend to cause upfield shifts of the adenine protons. At higher temperatures, though, Helene et al. (244) have noted that both the \( H_2 \) and \( H_8 \) protons of adenine experience a net upfield shift.
upon complex formation between polyA and 5-hydroxytryptamine (serotonin). In this case, adenine-adenine interactions have decreased considerably because of the higher temperature while the extent of complex formation, i.e. the amount of bound serotonin, has decreased only slightly; therefore since the indole ring system of serotonin has a strong magnetic anisotropy, the net shift of the adenine protons is determined by the upfield tendency of ring current interactions.

An intercalation model, with the aromatic side chains of the amino acids or peptides partially inserted between the bases of DNA or polyA, has been proposed to explain the experimental evidence (239). It is widely accepted that other planar aromatic molecules, such as the acridine dyes and ethidium bromide, form complexes with DNA by intercalation between adjacent base pairs (249). Insertion of the aromatic side chains between the bases of DNA or polyA ("sticky complex) would account for both the upfield shifts and peak broadening of the aromatic amino acid protons, due to ring-stacking with the nucleic acid bases and increased restriction of mobility of the aromatic side chains respectively. The temperature dependence of the changes in chemical shift of the adenine protons of polyA is also readily explained on the basis of the opposing effects (ring current interactions and disruption of interbase interactions) brought into play upon the intercalation of an aromatic moiety between adjacent adenine bases.

The extent of interaction of peptides containing aromatic amino acid residues with DNA base pairs appears to be dependent upon several factors.
It has already been noted that the different aromatic amino acids show different magnitudes of interaction in the decreasing order tryptophan > phenylalanine > histidine (236). As in the case of amino acid-nucleotide interactions, this order is probably determined in large part by the relative strengths of the ring current magnetic anisotropies for the aromatic amino acid side chains (246). Gabby et al. (238,248) have reported that there appears to be a dependence on the primary sequence of the peptide involved in complex formation when the aromatic residue involved is not tryptophan. A larger upfield chemical shift is observed for the aromatic protons if the aromatic residue is at the carboxyl-terminal end of a dipeptide or dipeptide amide in the case of phenylalanyl and tyrosyl residues. This seems to indicate that the aromatic rings of phenylalanine and tyrosine are in closer proximity to the base pairs of DNA when present at the carboxyl-terminal position.

In the case of dipeptide amides, this result might arise from an interaction of the positively charged \(-\text{NH}_3^+\) group with the phosphate anion on the periphery of the DNA helix, assuring that the carboxyl-terminal end of the dipeptide amide will be close to the helix (238). Tryptophan-containing peptides, perhaps because of tryptophan's stronger interaction with nucleic acid bases, show upfield chemical shifts which are independent of sequence (238,248).

As a third consideration, electrostatic contributions to the interaction between peptides containing aromatic residues and DNA are important. This is demonstrated by the dependence of the upfield shifts of the aromatic protons on ionic strength and pH. Dimicoli and Helene (250) have noted that the upfield shifts are decreased when either ionic strength or pH is...
raised, indicating a lessening of complex formation. The main factor involved seems to be the interaction of the peptide with the phosphate groups of the DNA helix. The presence of apolar amino acid residues or of amino acid residues containing an extra positive charge, e.g. lysine, in a dipeptide which contains an aromatic residue, enhances the upfield shift of the aromatic protons. However, the presence of two lysyl residues in a tripeptide amide decreases the interaction of an aromatic phenylalanyl residue with DNA (248) (as compared to the dipeptide with one lysyl residue), presumably due to a restricted binding geometry. This geometrical restriction involves the electrostatic interaction of the three positive charges with the phosphate anions, an interaction which hinders intimate contact between the phenyalanyl residue and the base pairs. Observed chemical shifts are also dependent on the relative concentrations of peptide and nucleic acid, and on temperature. (242).

Direct binding studies of dipeptide amides containing one lysyl residue and one aromatic amino acid residue with DNA by Gabbay et al. (248) suggest that these peptide amides may bind selectively to A-T sites, where the order of affinity to A-T binding sites is tryptophan > phenylalanine > tyrosine. The aromatic-containing peptide amides are more selective for A-T sites than those peptide amides with no aromatic residue. This selectivity may be the result of electronic (including ring current magnetic anisotropy) and/or steric factors; the mechanism for selectivity is not yet understood. As a consequence of the right-handed double helical nature of
DNA, with A-T and G-C base pairs, there are ten different intercalation sites in the helix. Each presents a slightly different environment to an intercalating molecule. The results of binding, absorption, and circular dichroism studies of complexes between DNA and intercalating "reporter molecules" by Gabbay et al (251) seem to strongly suggest that each of the ten possible intercalating sites has a different affinity for the intercalating molecules. Such a differentiation may be of great importance in the recognition process involved in protein-nucleic acid interactions.

There are a number of drawbacks to the study of interactions between aromatic amino acids or short peptides containing aromatic residues and nucleosides, nucleotides, or nucleic acids in aqueous solution. Low concentrations of the solutes are necessitated by solubility, ionic strength, and pH considerations and binding constants tend to be small. The net result is that complex formation is limited and significant amounts of each component remain free in solution. This complicates the study of the complexes by physical methods, since the results of the most informative measurements, such as nuclear magnetic resonance spectroscopy and fluorescence studies, reflect an average environment, taking into account both the free and the bound molecules, and low binding constants generally indicate that exchange between the forms will be rapid. It is then necessary to estimate the relative contributions of the bound and free states and make indirect calculations or extrapolations (usually necessitating simplifying assumptions which vary in accuracy) to determine the parameters of complex formation. Additional complications arise from the possibility of self-association of one or both components and from the coexistence of several complexes of different stoichiometries.
One way in which to avoid these problems in the study of protein-nucleic acid interactions is to use model compounds in which a protein fragment (an amino acid or short peptide) and a nucleic acid fragment (a nucleotide or oligonucleotide) are joined by a covalent bond (252). Use of such model compounds assures spatial proximity and facilitates interaction between an aromatic side chain and the nucleotide base. Even at low concentrations there is the potential for extensive interactions and the problems of unbound component molecules in the solution, self-association of unbound molecules, and the formation of several different complexes will be eliminated. Only intermolecular associations need be considered as a possible complicating factor and should cause no problem at low concentration.

Thus, nucleotide-peptides (compounds with a covalent bond between a nucleotide (or oligonucleotide) and an amino acid (peptide)) are beginning to receive growing attention. A number of nucleotide peptides are known to occur naturally and they can be isolated from various sources (253); they differ in nucleotide and peptide component chains and in the nature of the covalent linkage. Some of the intermediate structures formed in the course of transfer of amino acids (and possibly of nucleotides) are known to be nucleotide-peptides, and other molecules of this type may be responsible for structural arrangement of biopolymers (253), or may take part in cell metabolism (254).
Prior to a detailed study of the properties of nucleotide-peptides it was necessary to develop methods for the synthesis of these compounds. The primary factor involved is the choice of an effective means of activating the phosphoric acid residue of the nucleotide to promote the formation of a phosphoamide bond between the nucleotide and peptide fragments (253). Two methods have proven particularly effective - the pyrophosphate or mixed anhydrides method (255) and the carbodiimide method (254, 256).

The pyrophosphate method, adapted to the synthesis of nucleotide-peptides by Shabarova and coworkers (255), is based on using the highly reactive triesters of pyrophosphoric acids:

\[
\text{C}_6\text{H}_5\text{O}P\left(\text{O}R_1\right)_3 + \text{H}_3\text{COOC} - \text{CH} - \text{NH}_2 \rightarrow \text{R}_2 \text{H}_3\text{COOC} - \text{CH} - \text{NH}_2 \left(\text{P}\left(\text{OR}_1\right)_2\right)
\]

where \(R_1\) is a nucleoside and \(R_2\) is an amino acid side chain. The phosphoric acid residue of the nucleotide is activated by condensing the nucleotide with diphenylchlorophosphate in the presence of tri-n-butylamine in an inert, nonaqueous solvent (such as dioxane) to yield the \(1\)-nucleoside-\(5'(3')\)-\(2\)-diphenylpyrophosphate (compound I). Compound I, due to the stability of the diphenylphosphorinate anion, is a very active phosphorylating agent, and can be used to synthesize nucleotidyl-(P→N)- amino acids. The reaction between (I) and the amino acid ester is carried out in dioxane or dimethylformamide media for 12 hours at room temperature. Isolation and purification of the product, nucleotidyl-(P→N)- amino acid, is accomplished by preparative paper
chromatography. Yields for a number of nucleotidyl-(P\(\rightarrow\)N) -amino acids synthesized by the pyrophosphate method reportedly ranged from 28-95% (252,255).

A second method which has been adapted to the synthesis of nucleotide-peptides, again by Shabarova and coworkers (254,256), is the carbodiimide method, which has been widely used in peptide synthesis and in the synthesis of amides of nucleotides. The activation of the phosphoric acid residue of the nucleotide by N,N-dicyclohexylcarbodiimide (DCC) and the subsequent formation of the ester of the nucleotidyl-(P\(\rightarrow\)N)-amino acid (or peptide) in the presence of the ester of the amino acid probably occurs in the following way (256):

\[
\begin{align*}
R_1 - O - P - O^- + C_{6\text{H}_{11}}N &= C = NC_{6\text{H}_{11}} \\
&\text{(DCC)}
\end{align*}
\]

\[
\begin{align*}
R_1 - O - P - O - C_{6\text{H}_{11}}N &= C = NC_{6\text{H}_{11}} \\
&\text{(II) (Cyclohexylurea)}
\end{align*}
\]
The reaction take place in dimethylformamide over a period of three days at 37°C. Unfortunately, the high reactivity of the intermediate metaphosphate of the nucleoside (II) leads to a number of undesirable side reactions (256).

In a study by Shabarova, Gromova, and coworkers (257), nuclear magnetic resonance spectroscopy was used to study the intramolecular interactions in two nucleotide-amino acids (amines), adenylyl-(5'→N)-p-anisidine and adenylyl-(5'→N)-phenylalanine. They found that the influence of the adenine base on phenylalanine and anisidine, and vice versa, leads to readily observable changes in the NMR spectra of the two compounds. In both cases, the absorption lines of $H_2$ and $H_8$ of the adenine base are shifted upfield from the corresponding lines of adenosine-5'-phosphate. The ring protons of p-anisidine also showed upfield shifts in the NMR spectrum of adenylyl-(5'→N)-p-anisidine. The absorption of the ring protons of p-anisidine in N-(methylphospho)-p-anisidine consists of two closely spaced lines ($\delta = 7.17$ and $\delta = 7.20$); the corresponding absorptions in the spectrum of the nucleotidyl-amine appeared at $\delta = 6.51$ and $\delta = 6.78$ with spin-spin splitting constant $J = 8\text{Hz}$. The characteristic singlet of the phenylalanine ring protons ($\delta = 7.35$) was replaced by a complex multiplet in the spectrum of adenylyl-(5'→N)-phenylalanine at $\delta = 7.4$, due to the coupling of three types of protons; a significant portion of this multiplet was shifted upfield from $\delta = 7.35$. It appears likely that the upfield shifts of the aromatic protons are being caused by interaction between the ring current magnetic anisotropies of the adenine base and the benzene ring of phenylalanine.
or p-anisidine. Such interactions require close proximity of the two ring systems and a specific disposition in space of one ring with respect to the other; the NMR results are consistent with a coiled model of the nucleotide-amino acid (amine) which allows ring-stacking to occur between the adenine base and the aromatic amino acid (amine) residue with the two rings parallel and in close proximity to each other.
Our work this past year, which was aimed at developing such an understanding of the molecular basis of interactions between proteins and nucleic acids, went in two different directions. On the one hand, we attempted to synthesize a series of nucleotide-amino acid methyl esters in order to study nucleotide-amino acid interactions when the two species are brought into close proximity by being part of the same molecule. We also extended our previous studies of intermolecular interactions of model systems by concentrating on tryptophan and its interactions at low pD with dinucleoside phosphates containing adenine and cytosine residues.

1). Synthesis of a Nucleotide - Amino Acid Methyl Ester

The nucleotide amino acid methyl ester, adenylyl-5'(P→N)-L-tryptophan methyl ester, was synthesized by the carbodiimide method, as adapted by Ohtsuka et al (257) for the synthesis of N,2'-o-dibenzoylnucleoside-3-aromatic phosphoramidates and used by Shabarova and coworkers (258) for the synthesis of adenylyl-5'(P→N)-p-anisidine and adenylyl-5'(P→N)-phenylalanine. The following modifications were made: (1) reaction was allowed to take place for four hours at 82.5°C; (2) the progress of the reaction was followed by thin layer chromatography (TLC) on silica gel chromatograms in the solvent system, isopropanol: concentrated ammonia: water (7:1:2 by volume; the plates were spotted with reaction mixture every fifteen minutes; (3) carboxyamidine was removed from the 50% aqueous pyridine solution by slurrying this solution overnight with the pyridinium form of the cation.
exchange resin, AG50W-X8; (4) the aqueous pyridine solution was subsequently rendered anhydrous by stripping off that solvent using the rotary evaporator, followed by repeated additions of dry pyridine and evaporation to dryness.

The pyridinium form of cation exchange resin was prepared by slurrying the hydrogen form of the cation exchange resin AG50W-X8 with pyridine for several days. Excess pyridine was removed by repeated washing with deionized water. The hydroxide form of anion exchange resin was prepared by slurrying the chloride form of AG1-X8 with 1N NaOH for several days.

The dipyridinium salt of adenosine-5'-monophosphate (5'AMP) was prepared from adenosine-5'-monophosphoric disodium, trihydrate by slurrying the disodium salt with the pyridinium form of the cation exchange resin overnight (at refrigerated temperature). The aqueous solution of adenosine-5'-monophosphoric dipyridinium (pyridinium AMP) was washed from the resin with deionized water and concentrated on the rotary evaporator. The UV and NMR spectra of pyridinium AMP were recorded.

L-tryptophan methyl ester (L-trp-OMe) was prepared from L-trp-Ome·HCl by a modification of the procedure of Fisher and Suzuke (259) as detailed by Pirkle and Beare (260). Due to the relatively low solubility of L-trp-Ome·HCl in absolute methanol, the solution was heated to just below the boiling point of methanol, and additional absolute methanol was added until all of the L-trp-Ome·HCl dissolved. This required two to three times the stated volume of absolute methanol. L-trp-OMe was identified by its NMR spectrum, as given by Pirkle and Beare (260).
The components of the reaction product mixture remaining after the initial purification steps (259) were separated by preparative descending chromatography on Whatman grade #3 chromatography paper using the solvent system isopropanol: concentrated ammonia: water (7:1:2 by volume). The bands were eluted using the above solvent system or absolute methanol. This solvent system was used by Shabarova and coworkers in characterizing adenylyl-5' (P → N)-L-tryptophan methyl ester (adenylyl-5' (P → N)-L-trp-OMe) (252) and in the purification and isolation of a number of nucleotide-amino acids (254-256). The procedure for descending paper chromatography described by Block et al (261) was followed.

Each of the starting reagents was characterized by its ultraviolet-visible spectrum and its nuclear magnetic resonance spectrum, with special interest in the aromatic region (approximately $\delta =6$ to $\delta =9$). TMS and tert-BuOH were used as internal reference standards; all chemical shifts, $\delta$, were determined relative to TMS. The methyl peak of tert-BuOh occurs at $\delta =1.28$ relative to TMS (262).

Adenylyl-5' (P → N)-L-tryptophan methyl ester was chosen as the object of our studies because if ring-stacking does occur between the nucleotide base and the aromatic side chain of a nucleotide-amino acid, then adenosine and tryptophan should each induce the largest possible upfield shifts for this type of interaction in the NMR peaks of the aromatic protons of the other fragment in the stacked pairs. This expectation is based on the calculations of ring current intensities and intermolecular shielding values for the nucleotide bases and the aromatic amino acids by Giessner-Prette and Pullman (240,247,263). The results of their calculations indicated that only adenosine,
of the nucleotide bases, and tryptophan, among the aromatic amino acids, could be expected to induce upfield shifts as great or greater than 1 ppm in the protons of neighboring molecules. Large upfield shifts of the aromatic resonance peaks of both components would, of course, make the nuclear magnetic resonance study of intramolecular ring-stacking interactions in adenylyl-5' (P → N) -L-try-OMe easier and more informative. The results of optical rotatory dispersion and circular dichroism studies of adenylyl-5' (P → N) -L-trp-OMe by Shabarova and coworkers (252) indicate that such intramolecular ring-stacking interactions do occur in this molecule.

The synthesis of adenylyl-5' (P → N) -L-trp-OMe was carried out for the first time in 1971 (252) by the pyrophosphate method described earlier for nucleotide-amino acids. It was decided, however, to try an adaptation of the carbodiimide method developed by Ohtsuka et al (257) and used by Shabarova and coworkers (258) for the synthesis of adenylyl-5' (P → N) -p-anisidine and adenylyl-5' (P → N) -phenylalanine. The advantages of this method over the pyrophosphate method and the carbodiimide method used previously in the synthesis of nucleotide-amino acids (254, 256) are:

(1) "nearly quantitative" yield (257) (Shabarova and coworkers (258) reported yields of 95 to 97% from their synthesis using this method); (2) a considerably shorter reaction time, this being an important consideration in view of the fact that many of the nucleotide-amino acids decompose relatively rapidly upon storage (254, 256); (3) the use of 80% aqueous tert-BuOH which eliminated the necessity for a rigorously dry reaction system which is required by the other two methods, and hence greatly simplifies the synthetic procedure.
The starting materials required for the synthesis by the modified carbodiimide method were L-tryptophan methyl ester, adenosine-5'-monophosphoric dipyridinium, and N,N'-dicyclohexylcarbodiimide. Difficulties were encountered in preparing L-trp-OMe and pyridinium AMP from the available reagents, L-tryptophan methyl ester, hydrochloride and adenosine-5'-monophosphoric disodium, trihydrate.

The conversion of the hydrochloride of L-trp-OMe to the free amino acid methyl ester was initially attempted through the use of an anion exchange resin (AG1-X8) in its hydroxide form: the L-trp-OMe·HCl was slurried over night with the resin in an aqueous solution at refrigerated temperature. However, it proved impossible to wash most of the L-trp-OMe from the resin with water, absolute methanol, or ethanol, even when these solvents were heated. Apparently the amino acid methyl ester remains attached to the resin or precipitates in the resin when Cl⁻ is exchanged for OH⁻ and its limited solubility makes it difficult to subsequently free L-trp-OMe from the resin.

The next approach to the preparation of L-trp-OMe was an attempt to precipitate the free amino acid methyl ester by titrating L-trp-OMe·HCl with the weak base, bicarbonate. A quantitative titration, however, did not cause precipitation of L-trp-OMe, neither did adding additional bicarbonate nor chilling the titrated solution in an ice bath. Titrating with the strong base, NaOH, did result in the formation of white precipitate, which subsequently oiled and finally went into solution (this solution
being yellow in color) with the addition of more base. An NMR spectrum of
the resulting solution strongly suggested (when compared to the spectrum
of L-trp-OMe (260)) that the free amino acid methyl ester had not been
prepared but that some other reaction had taken place.

It is likely that a cyclization of L-trp-Ome had taken place:

\[
\begin{align*}
\text{L-trp-Ome} & \quad \rightarrow \quad \text{Cyclization Product} \\
\end{align*}
\]

A space-filling model of the proposed cyclization product was very strained
by the formation of the new six-membered ring. This is probably the result
of the presence of a bridge-head double bond between the five-membered ring
of the indole system and the new ring. It seems unlikely, in view of the
observed molecular strain, that the proposed cyclization would not be a
stable one. No other model has been proposed to account for the reaction
caused by the addition of NaOH to L-trp-OMe·HCl.

The method of Pirkle and Beare (260) for the preparation of free
amino acid methyl esters from the amino acid methyl ester hydrochlorides was
used successfully to prepare L-trp-OMe with the modification mentioned previously.

The method involves the treatment of the amino acid methyl ester
hydrochloride with sodium methoxide in a solution of absolute methanol followed by the addition of ether to precipitate salts. The L-trp-OMe, evaporated to dryness on the rotary evaporator, was a pale yellow crystalline solid and was used without further purification; the product yield was approximately 30%.

The synthesis of adenosine-5'-monophosphoric dipyridinium (pyridinium AMP) involved problems also. The aqueous solution containing what was believed to be pyridinium AMP (a UV spectrum of the solution showed a strong absorption band at 257 nm with shoulders at 250 and 262 nm and was interpreted as an overlapping of the absorptions due to pyridinium and AMP) was filtered from the pyridinium cation exchange resin and evaporated to dryness. A UV spectrum of the white crystalline product, however, showed only a strong absorption maximum at 269 nm, and its NMR spectrum in D₂O showed the same resonance peaks recorded for Na₂AMP·3H₂O. In view of these similarities between the UV and NMR spectra of "pyridinium AMP" and Na₂AMP·3H₂O, especially since pyridine is known to have strong UV absorption and characteristic NMR resonance peaks in the aromatic region, it seems probable that a large proportion the Na₂AMP·3H₂O did not exchange cations. The "pyridinium AMP" prepared in this way was used as a starting material for the syntheses in spite of the doubt as to the nature of its cations. The pyridinium salt was desired as a starting material because of the relative ease with which excess pyridinium ions would be removed with the solvent (aqueous pyridine) during the purification steps as compared to the sodium ions. However, there
was no observed hindrance to the reaction due to the possible presence of sodium ions. More than 25% of the "pyridinium AMP" was not recovered from the pyridinium resin.

The third starting reagent, N,N'-dicyclohexylcarbodiimide, was used as purchased, without further purification. The DCC was pulverized before addition to the reaction mixture in order to increase the surface area accessible to the solution.

Several modifications of the carbodiimide method used by Shabarova and coworkers (258) were made for this synthesis of adenylyl-5'(P → N)-tryptophan methyl ester. (1) The recommended temperature and duration of heating of the reaction mixture were 90° to 100°C and two hours respectively. We found, however, that a solution of 80% tert-BuOH and 20% H₂O is constant boiling at 82.5°C. As it was desired to reflux the reaction mixture during heating rather than carry out the reaction in a sealed tube, it was decided to run the reaction for four hours at 82.5°C.

(2) The progress of the reaction was checked at fifteen-minute intervals by thin layer chromatography (silica gel) rather than by electrophoresis. Thin-layer chromatography was considered to be a convenient method and it had been demonstrated that a good separation of the starting materials, pyridinium AMP and L-trp-OMe, could be achieved on silica gel. It was later found that a better separation could be achieved on paper and on cellulose thin-layer chromatograms than on silica gel: thus, cellulose chromatograms were used to monitor part of the second synthesis. The only disadvantage of this thin-layer chromatography monitoring system was that the time lag due to the long development time (over two hours for each
chromatogram) made it impossible to know the state of progress of the reaction at any given time of its duration.

(3) The 50% aqueous pyridine solution in which the reaction mixture residue was dissolved was slurried with a pyridinium cation exchange resin over night at refrigerated temperature instead of being passed down a column of the exchange resin. This change was made on the basis of finding a slurry more convenient to work with than a column. However, because of the rather large volume of aqueous pyridine required to dissolve the residue (approximately 200 ml), it is possible that following the original procedure would give more efficient results.

In its final form the synthesis of adenylyl-5' (P → N)-L-tryptophan methyl ester by the modified carbodiimide method ran as follows:

1. Prepare L-trp-OMe from L-trp-OMe·HCl, by the method of Pirkle and Beare, evaporating ether solution to dryness to obtain the amino acid methyl ester in crystalline form.

2. Prepare pyridinium AMP by slurrying Na₂AMP·3H₂O over night with pyridinium cation exchange resin at refrigerated temperature; evaporate aqueous filtrate from slurry to dryness on the rotary evaporator.

3. Add 1 mmole of pyridinium AMP, 7 mmoles of L-trp-OMe and 5 mmoles of DCC (pulverized) to a solution of 2 ml H₂O and 8 ml tert-BuOH.

4. Reflux reaction mixture at 82.5°C for four hours, checking progress every fifteen minutes by thin-layer chromatography.

5. Concentrate reaction mixture to a gum using the rotary evaporator.

6. Dissolve gum in 50% aqueous pyridine.

7. Extract with n-hexane three times.
8. Filter to remove cyclohexylurea.

9. Equilibrate filtrate with slurry of pyridinium cation exchange resin to remove "carboxyamidine".

10. Render solution anhydrous by stripping off the aqueous pyridine with the rotary evaporator, followed by repeated additions of dry pyridine and evaporation to dryness.

11. Dissolve residue in pyridine.

12. Add solution to a mixture of ether (200 ml) and n-hexane (100 ml) under vigorous stirring.

13. Collect precipitate by centrifugation and wash with ether three times.

A problem encountered frequently in this synthesis of adenylyl-5' (P \rightarrow N ) - L-tryptophan methyl ester was that of poor solubility. This has already been mentioned with respect to the preparation of L-trp-OMe from L-trp-OMe·HCL. None of the three starting materials, L-trp-OMe, pyridinium AMP, and DCC, dissolved completely in the 80% tert-BuOH/20% H_2O solvent system; the reaction mixture was initially faint yellow in color and quite viscous with undissolved reagents. During the four-hour course of the reaction, a large proportion of the reagents seemed to dissolve (some undissolved particles were always evident at the bottom of the flask) and the reaction mixture became progressively darker yellow in color, remaining rather viscous. A fairly large volume of 50% aqueous pyridine (150-200 ml) was required to dissolve the reaction mixture residue after its concentration to a gum; this solution was amber (dark red/brown when concentrated) in color.
The precipitate collected in the last step of the synthesis (formed upon addition of the pyridine solution of the reaction product mixture to the ether-hexane mixture) was flaky and beige in color, but formed bright amber solutions in pyridine, methanol and dimethylsulfoxide (DMSO). The product mixture was negligibly soluble in water (and D₂O), chloroform (and CDCl₃), and carbon tetrachloride, slightly soluble in methanol (and methyl alcohol d₄), and readily soluble in pyridine and DMSO. DMSO was not considered a good solvent for NMR studies of the product mixture, however, as it is known to destack aromatic rings in solution (264).

The desired reaction is shown below with A = adenosine and R = tryptophan side chain:
Some possible side products (as discussed earlier in connection with the carbodiimide method) are shown below. The large excess of L-trp-OMe and of DCC used will tend to minimize the production of the symmetrical diadenosine pyrophosphate and the adenosine dinucleotide, but may be expected to increase the side reactions leading to the synthesis of the tryptophan derivatives of DCC and of the symmetrical diadenosine pyrophosphate.

\[
\begin{align*}
\text{(i)} & \quad A - O - P - P - O - A \\
\text{Symmetrical diadenosine pyrophosphate} \\
\text{(ii)} & \quad A - O - P - O - A \\
\text{Adenosine dinucleotide} \\
\text{(iii)} & \quad A - O - P - O - A \\
\text{Tryptophan derivative of (i)} \\
\text{(iv)} & \quad C_6H_{11}N \quad C \quad N \quad C_6H_{11} \\
\text{Tryptophan derivative of DCC "Carboxyamidine"}
\end{align*}
\]

\[A = \text{adenosine residue} \]
\[R = \text{tryptophan side chain}\]

Possible side products in synthesis of adenylyl-5'(P → N)-L-tryptophan methyl ester.

A space-filling model was assembled for the desired product, adenylyl-5'(P → N)-L-tryptophan methyl ester, in order to study its possible conformations.
It is possible to create a large number of conformations in which close ring-stacking can occur between adenine and the indole ring system of tryptophan. It should be noted that there is also a clear possibility of intramolecular hydrogen bonding. A factor such as intramolecular hydrogen bonding could be of great importance in providing thermodynamic stabilization to a given conformation, and, furthermore, could be detected via NMR spectroscopy.

Spectral Interpretation - Ultraviolet-Visible and Nuclear Magnetic Resonance. The starting materials were characterized by their UV and NMR spectra and, where possible, the values for absorption maxima and chemical shifts were compared to literature values. In the UV region, the absorption due to two chromophores - the heterocyclic base, adenine, and the indole ring system of L-tryptophan - is of particular interest, as both these chromophores occur in the desired product, adenylyl-5'-(P→N)-L-trp-OMe, and their absorptions are easily distinguishable. In the NMR spectra, the resonance peaks of the aromatic protons on adenine and on the indole ring system of tryptophan are of greatest interest because it is these resonance peaks that should experience significant upfield shifts if ring-stacking interactions do take place in the model system nucleotide-amino acid methyl ester. In addition, the positions of other, possibly hydrogen-bonded, proton peaks are important.

The UV spectrum of the reaction product mixture after initial purification steps exhibits an absorption maximum at 267 nm with a weak shoulder at 278 nm and a sharp shoulder peak at 289 nm. This absorption
pattern indicated that there are both adenine and tryptophan residues present in the product mixture. The appearance of a new low-intensity absorption band starting at approximately 320 nm and tailing off in the visible region around 550 nm might indicate the presence of a charge-transfer complex in the product mixture (none of the starting materials showed absorption in this region). This possibility is supported by the observation that although the product mixture is beige in color (possible due to the presence of a colored contaminant), a solution of the product mixture in pyridine and methanol is bright amber in color. The existence of charge-transfer complexes due to ring-stacking between an aromatic amino acid residue donor and a purine or pyrimidine base acceptor has been reported in frozen aqueous solutions (265,266). In a recent study, Herriott et al (267) have discussed the possibility that the model compound 1-(2-indol-3-ylethyl)-3-carbamidopyridinium chloride (an intramolecular model of the nicotinamide adenine dinucleotide-tryptophan charge-transfer complex) exists in a folded conformation in solution that allows for a ring-stacking between the indole ring system and the pyridinium residue. Thus, it is possible that ring-stacking in adenyl-5'(P\(\rightarrow\)N)-L-trp-OMe would also result in a charge-transfer interaction which could result in the appearance of a new absorption band at longer wavelengths (265,266). The UV spectrum of the product reaction mixture, then, gives the first indication that some reaction has taken place and that the presence of both adenine and tryptophan residues (as indicated by the absorption pattern) does not indicate just a mixture of the starting materials (the UV spectrum of a mixture of Na\(_2\)AMP·3H\(_2\)O and L-trp-OMe·HCl shows no absorption in the region of 320-550 nm).
2). **Intramolecular Interactions of Amidates**

The NMR spectrum of the reaction product mixture in $D_2O$ showed absorption in the aromatic region from $\delta = 7$ to $\delta = 7.5$, with one distinct peak appearing $\delta = 7.3$. However, the intensity was too low and the resolution too poor to make any interpretation. The NMR spectrum of the product mixture in methanol was more interesting. There were three regions to be noted: (1) a broad multiplet from approximately $\delta = 4.6$ to $\delta = 5.7$ with two distinct peaks at $\delta = 4.85$ and $\delta = 5.05$; (2) two small peaks at $\delta = 5.95$ and $\delta = 6.30$; (3) a poorly resolved multiplet from $\delta = 7.85$ to $\delta = 8.45$. One possible interpretation of the observed peaks is that the three regions listed above correspond respectively to (1) the aromatic protons of the indole ring system of tryptophan methyl ester residues involved in ring-stacking and shifted upfield, (2) the $H_2$ and $H_8$ peaks of AMP residues, involved in ring-stacking and shifted upfield, and (3) the $H_2$ and $H_8$ peaks of side products containing AMP residues not involved in ring-stacking and not shifted. This would mean, however, that the aromatic resonance peaks of both tryptophan and adenosine have been shifted upfield by 2ppm, twice as much as the maximum upfield shift likely to be induced by either adenine or tryptophan according to the calculations of Giessner-Prettre and Pullman (245,247). Even with the 20% increase in intramolecular shielding values recommended by Shulman and coworkers (268,269), the upfield shifts would be much larger than predicted on the basis of ring current intensities. Without better resolution, and in view of the impurities in the reaction product mixture, it is not possible to make a conclusive judgement on the basis of this spectrum.
3. **Intermolecular Interactions Between Tryptophan and Dinucleoside Phosphates at low pD**

As described in the previous section, tryptophan, because of its considered ring current magnetic anisotropy, can induce large upfield shifts of the proton magnetic resonances of heterocyclic bases in such molecules as adenosine and cytidine. Similarly, adenine and cytosine each are capable of inducing upfield shifts in the resonances of protons on other ring systems. The stoichiometry and geometry of the interaction of tryptophan with dinucleoside phosphates was investigated by monitoring upfield shifts in the proton magnetic resonance (pmr) spectra of tryptophan and the dinucleoside phosphates adenylyl-3'→5' - adenosine (ApA), cytidylyl - 3'→5' - cytidine (CpC), adenylyl - 3'→5' - cytidine (ApC) and cytidylyl-3'→5' - adenosine (ApC) when they are dissolved together in solutions at low pD (0.1 N DCl in D₂O).

Low pD is required for the solubility of tryptophan to be sufficient for pmr study. The effects of this low pD must be taken into account when considering the results obtained. Since it was anticipated that the highly acidic solution might cause breakdown of the dinucleoside phosphates, solutions were tested, immediately after pmr spectra were recorded, by thin layer chromatography. Thus, it was demonstrated that no breakdown occurred, and it may be concluded that the pmr spectra obtained were due only to intramolecular interactions between the dinucleoside phosphates and tryptophan.

Initially, pmr spectra were recorded on a continuous wave 100 MHz.
instrument, a Varian AA-100, at Harvard University, operating in the frequency sweep mode. Recent acquisition by this laboratory of a Varian CFT-20 adapted for protons, i.e. a fourier-transform 80 MHz instrument is now providing spectral results of greater precision and clarity in far more convenient form and in less time than were available with the continuous wave instrument.

The experimental procedure followed involved producing solutions containing various ratios of tryptophan to mono- or dinucleoside phosphate by directly adding specific volumes of tryptophan solution to known volumes of mono- or dinucleoside phosphate solution in a pmr sample tube. Since all stock solutions were of approximately the same concentration, the total concentration of all species in a mixture was always the same. Dilution series were also done of tryptophan itself and of individual mono- or dinucleoside phosphates.
Pmr spectra were recorded at 32.5° ± 1.00 on a Varian HA-100 nuclear Magnetic Resonance Spectrometer operating in the frequency sweep mode. Typical peak resolution was < 1Hz at half height.

Immediately after each spectrum was recorded, a 1 µl sample was removed from the pmr tube and applied to a silica gel plate which was subsequently developed in the previously mentioned solvent system. This allowed the monitoring of the compounds under study under the conditions of the experiment. No evidence of impurity or breakdown of any compound was observed.

**Dilution Effects**

Resonance positions of aromatic protons were determined from pmr spectra measured for the following compounds in the following concentration ranges in 0.10N DCl in D₂O: adenosine 5' monophosphate (5'AMP), cytidine 5' mono-phosphate (5'CMP), and adenylyl (3'→5') adenosine (ApA): 0.10m to 0.025m; cytidylyl (3'→5') adenosine (CpA): 0.087m to 0.022m; adenylyl (3' 5') cytidine (ApC): 0.078m to 0.02m; cytidylyl (3'→5') cytidine (CpC): 0.12m to 0.03m; tryptophan (Trp), 0.10m to 0.02m.

1. **Nucleoside Monophosphate Dilution Studies**

![L-tryptophan](image)

![Adenine](image)

![Cytosine](image)
The H-2 and H-8 peaks of 5' AMP (see above) moved progressively downfield as the AMP was diluted. The downfield shift was most pronounced in the range between 0.10M and 0.05M, and was greater for the H-2 peak than the H-8 peak.

The H-5 and H-6 peaks of 5' CMP were similarly affected by dilution, although the magnitude of the effect was less than that observed for the AMP protons. Most of the downfield shift was observed in the concentration range 0.10M to 0.07M, with the peak positions remaining fairly constant at concentrations lower than 0.07M.

2. Dinucleoside Monophosphate Dilution Studies

The H-2 and H-8 peaks of both adenine residues of ApA showed no appreciable change in position with dilution over the concentration range studied.

CpA showed weak dilution effects in the form of downfield shifts of the H-2 and H-8 resonances of the adenine residue and the H-5 and H-6 resonances of the cytosine residue for the concentration range of 0.087M to 0.066M. The effect was greater for the resonances of adenine than for those of cytosine. No apparent dilution effects were observed for any of the protons between 0.066 and 0.022M.

Upon dilution of ApC, the pmr spectra exhibited downfield shifts of the H-2 and H-8 adenine proton resonances and the H-5 and H-6 cytosine proton resonances of a magnitude similar to that of the H-8 proton of 5' AMP. The effect is greatest between 0.078M and 0.05M and negligible
between 0.05M and 0.02M. The effect of dilution on the H-2 (adenine) and H-5 (cytosine) resonances is greater than that on the H-8 (adenine) and H-6 (cytosine) resonances.

Dilution had no appreciable effects on the H-5 or (and) H-6 resonances of either cytosine residue of CpC.

3. Tryptophan Dilution Studies

Five representative peaks chosen from the aromatic portion of the pmr spectra of tryptophan showed no appreciable downfield shift upon dilution in the range studies (0.1OM to 0.02M).

Effects of Addition of Tryptophan to Solutions of mono- and Dinucleoside Phosphates

To each solution of mono- or dinucleoside phosphate (concentration .1M), varying amounts of tryptophan solution (also .1M) were added. This allowed the ratio of the concentrations of the components to vary, but kept the sum of their concentrations constant (within + 0.005M for each mixing series).

The changes in the mono- or dinucleoside phosphate spectra resulting from the addition of tryptophan were determined and then corrected for dilution effects by measuring the difference in peak position between a mono- (M) or dinucleoside phosphate (DNP) at a given concentration in the presence of tryptophan and a M or DNP at the same concentration in the absence of tryptophan (as shown by the dilution curve). In all cases, the presence of tryptophan induced upfield shifts of aromatic proton resonances in the M and DNP's. Similarly, in all cases, the M and DNP's caused upfield shifts of aromatic proton resonances in the spectra of tryptophan.
1. Mononucleoside phosphates

Addition of tryptophan to 5' AMP caused upfield shifts of the H-2 and H-8 resonances. The shift induced in the H-2 resonance was about three times the shift found for the H-8 resonance. For both protons, the rate of change of resonance position as the tryptophan/5'AMP ratio was increased was greatest when tryptophan was present in less than a 1:1 ratio.

In 5' CMP, the addition of tryptophan caused upfield shifts in H-5 and H-6 resonances. The shift induced in the H-5 resonance was about twice that induced in the H-6 resonance. In both cases, the rate of change of resonance position was almost constant as the ratio of trp to CMP increased.

2. Dinucleoside phosphates

ApA Tryptophan induced upfield shifts in the H-8 and H-2 resonance peaks of both adenine residues of ApA. The H-2 resonances (which are shifted equally) were shifted more than twice as much as the H-8 resonances. In both cases, the shifts did not increase above a Trp/ApA ratio of about 2.5:1. The H-8 resonances for both adenine residues appeared as one peak below a Trp/ApA ratio of about 3/4:1. At this point, the two peaks became resolved, one about 2Hz upfield of the other. They remained within 2Hz of each other as they were shifted further upfield by the addition of more tryptophan.
The addition of tryptophan to CpA induced upfield shifts in the resonances of H-5 and H-6 of the 3' cytosine residue and H-2 and H-8 of the 5' adenine residue. In all cases there was a marked increase in upfield shift as the Trp/CpA ratio was increased to 1/2:1. Above this ratio the rate of increase in shift with increase in ratio was constant for each proton resonance. Below the 1/2:1 ratio, H-8 (5') and H-6 (3') resonances underwent shifts of approximately the same magnitude. The H-2 (A) resonance was shifted about twice as much, and the H-5 (C) resonance was shifted slightly less than that of H-2 (5'). Above the ratio of 1/2:1, the constant rate of increase in shift was approximately equal for the resonances of H-8, H-6, and H-5, while the rate for H-2 was about twice that of the other protons.

Adding tryptophan to ApC caused upfield shifts in the resonance peaks of H-2 (3' adenosine residue), H-8 (3' adenosine residue), H-5 (5' cytidine residue), and H-6 (5' cytidine residue). The H-8 (3'A), H-5 (5'C) and H-6 (5'C) resonances all experienced comparable sharp increases in upfield shift as the Trp/ApC ratio increased from 0 to 1/2:1. At higher ratios, no further upfield shifts were found. The H-2 (3'A) resonance was shifted about twice as far upfield as the other three resonances. The shift H-2(3'A) increased as the Trp/ApC ratio increased from 0 to 3/4:1, then remained nearly constant as the ratio increased beyond that point.
Tryptophan induced upfield shifts in the H-6 and H-5 resonances of both cytosine residues of CpC. Before the tryptophan was added, the two H-6 proton resonances appeared as one peak. As soon as the tryptophan was added, the protons appeared as separate peaks. The distance between these peaks increased from zero to three Hz as the Trp/CpC ratio increased from 0 to 1:1. The separation between the peaks remained at 3Hz at all ratios above 1:1. The shift induced in all four resonances monitored increased gradually as the Trp/CpC ratio increased from 0 to 1:1. The shift induced in the downfield H-6 resonances was slightly less than that induced in the upfield H-6 resonance and in the two H-5 resonances. The shifts of the two H-6 resonances and the 5' H-5 resonance increased only slightly at ratios above 1:1. The 3' H-5 showed gradually increasing shifts up to a ratio of about 2:1, and a nearly constant shift at higher ratios.

3) Comparison of Upfield Shifts of Corresponding Protons of Different Mono- and Dinucleoside Phosphates Upon Addition of Tryptophan

Adenine H-8

The greatest overall upfield shift of the H-8 proton resonance occurred in the (5') H-8 of CpA. Only slightly smaller than that was the shift of the 5' H-8 of 5'AMP. Smaller still, and all of comparable size, were the shifts of the 5' H-8 of ApA, the 3' H-8 of ApA and the 3'-H-8 of ApC.

The shifts of the 5' H-8 of CpA and the 3' H-8 of ApC show sharp increases at low Trp concentrations. The H-8 resonance of both adenine residues of ApA...
and of AMP show more gradual increases at the low Trp concentrations.

**Adenine H-2**

The greatest overall upfield shifts, of equal magnitude, occurred for the 5'H-2 resonance of 5' AMP and CpA. The shifts of the two H-2 protons in ApA were about 2/3 as great as those for 5' AMP and CpA, while the overall shift of the 3' H-2 of ApC was slightly smaller than that of the ApA protons H-2.

As with the H-8 protons, the H-2 protons associated with CpA and ApC experienced sharp increases in shift at low Trp concentrations, while the other H-2 protons experienced more gradual increases.

**Cytosine H-6**

The largest overall upfield shift for an H-6 resonance in the series studied was associated with the 3' H-6 of CpA. Somewhat smaller and unequal to each other were the shifts of the two H-6 protons of CpC. The 5' H-6 proton of ApC experienced an overall shift of approximately the same magnitude as the one experienced by the less shifted of the two CpC H-6 protons. The 5'H-6 of 5'CMP experienced an even smaller shift.

Once again, the protons associated with ApC and CpA underwent sharper increases in shift at low Trp concentrations than did protons of the other compounds studied.
Cytosine H-5

The largest overall shift was experienced by the 3' H-5 of CpA. Somewhat smaller were the shifts of the 3' H-5 and 5' H-5 of CpC, which were comparable to each other. The shift associated with the 5' H-5 of 5' CMP was slightly smaller, and smaller still was the overall shift experienced by the 5' H-5 in ApC.

As before, protons associated with ApC and CpA experienced the sharpest increases in shift at low Trp concentrations.

4). Effects of MNP's and DNP's on Tryptophan


In general, the singlet peak of H-2 in the 5'-membered ring, is shifted less than the peaks associated with the 6'-membered ring. In two cases (5'AMP and ApC) the selected peak furthest downfield is shifted slightly less overall than the H-2 peak.

CpC and 5' CMP caused only very small upfield shifts in the tryptophan peaks, and all shifts were of roughly comparable magnitude.
In analyzing our results, the influences of the conditions which were used must be taken into consideration. The first of these is the acidic nature of the solutions. In 0.1N DCl the adenine and cytosine rings of both the nucleotides and the dinucleoside monophosphates are protonated (270) as is the phosphate group itself. In addition, the tryptophan is in the cationic form. Protonation of nucleosides and nucleotides has been shown by Danyluk and Hruska to cause downfield shifts of the resonance peaks assigned to the ring protons (272). They attribute this deshielding of the ring protons primarily to a redistribution of π-electron densities in the heterocyclic rings. Chan and Nelson (271), in their study of adenylyladenosine, also noted a downfield shift of all four ring proton resonances with decreasing pH. At pH 4, they postulate that only one base of the dinucleotide is protonated. Both bases are monoprotonated below pH 3, and at pH 0 the phosphate group is also protonated. The relative positions of the resonance peaks of the nucleotides and dinucleoside monophosphates obtained by us are in good agreement with those reported by Ts'o et al (273) and by Chan and Nelson (271). In addition to the downfield shifts resulting directly from protonation of the bases, a second important effect noted by Chan and Nelson (271) is the breakdown of basestacked arrays due to electrostatic repulsions between bases. Whereas stacking interactions are quite significant at neutral pH when there are no charges on the rings, Chan and Nelson (271) have demonstrated that, at pH 1, the two adenine rings of ApA are destacked. These findings would be expected to apply as well to the effect of protonation on intra- and intermolecular interactions involving CpA and to intermolecular interactions among adenosine monophosphates or cytidine monophosphates.
Amino acid protons also exhibit changes in chemical shifts when pH is lowered. According to Roberts and Jardetzky (274), C\textalpha{}H resonances are shifted downfield about 0.3 ppm on going from the zwitterionic form to the cationic form. The C\textalpha{}H peaks are also shifted slightly downfield by amounts which depend on the amino acid. For the aromatic amino acids, these shifts range from 0 to 0.19 ppm. The ring proton resonances are not significantly shifted on protonation of the carboxyl group. The assignments of the tryptophan resonances given in this paper are based on the chemical shifts given by Roberts and Jardetzky (274).

The second experimental condition which might be expected to influence our results is the use of high concentrations of solutes, which increases the likelihood of intermolecular interactions among like molecules. As discussed above, this tendency is offset, in the case of the nucleotides, by the electrostatic repulsion arising from protonation of the bases in the acidic medium.

The changes in the chemical shifts observed when adenosine monophosphate or cytidine monophosphate is mixed with tryptophan demonstrate the presence of intermolecular interactions between the nucleotides and the amino acid. Information about the relative orientations of the molecules in the complexes may be obtained from a more detailed analysis of the results. The fact that the upfield shift of the H-2 resonance of adenosine is nearly three times that of the H-8 resonance, may be considered evidence that the stacking of adenosine with tryptophan primarily involves the six-membered ring of the nucleoside. Similarly, upfield shifts of the H-5,6 and H-4,7 resonances of
tryptophan in the presence of adenosine monophosphate are about twice that of the H-2 resonance. Thus, the complex is most likely formed by the overlap of the six-membered rings of tryptophan and adenosine.

The interaction of tryptophan with cytidine monophosphate may be interpreted in a similar manner. In this case the greater upfield shift for the H-5 of the cytosine ring suggests a geometry of interaction with tryptophan such that this proton is closer to the most significant area of tryptophan ring current magnetic anisotropy. The effect of cytidine monophosphate on the tryptophan spectrum is negligible, as can be predicted from the small ring current magnetic anisotropy of the cytosine ring.
REFERENCES


151. Perry, R.P., Personal Communication.
III. TIME AND EFFORT

During the past year the principal investigator has spent about 70% of his time on the projects outlined in this progress report. He will continue to spend about 70% of his time and effort on these projects during the remainder of the current term of the agreement.
IV. PUBLICATIONS AND REPORTS

Since the previous progress report the following publications reporting on the work described in this progress report and previous progress reports have appeared.


The following papers based on the work described in this and previous progress reports have been accepted for publication and are in press.


Kolodny, G.M. The Regulation of Gene Expression in Eukaryotic Cells, Medical Hypothesis

Kolodny, G.M. Turnover of Ribosomal RNA in Mouse Fibroblasts (3T3) in Culture Exp. Cell Res.

The following manuscripts based on the work described in previous progress reports have been submitted for publication.


Kolodny, G.M., Methylation Pattern During Turnover of Ribosomal RNA from Mouse Fibroblasts (3T3), Biochim. Biophys. Acta.

The following manuscripts based on work described in previous progress report are now being prepared for submission.


As an invited conference participant, manuscripts solicited for the proceedings to be published by the following conferences:

Kolodny, G.M.: A Possible Role in Gene Regulation of RNA from the same or Different Cells. Int. Pitisee Conference on "Information Transfer between Cells" April 1972, Pitisee/Black Forest, Germany.
PROGRESS REPORT:

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by

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