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MOLECULAR EVENTS BASIC TO
CELLULAR RADIATION RESPONSE

by

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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 (G_1 or G_0) 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 G_1 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 G_1 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 by changes in gene expression, the the initiating trigger for cell division is probably found in the activation of one of 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 genes 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 (26,27). 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), 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 (29). We found a single component decay curve if the cells were labeled after confluence was attained, similar to the results of others (30). 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, since 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 investigated turnover of ribosomal RNA seeking direct evidence for the reutilization of oligonucleotide segments en bloc of either the 18s or 28s RNA in the synthesis of new ribosomal RNA.

II. REPORTS

A. Regulation of Gene Expression

1) Turnover of Ribosomal RNA

We have presented a new hypothesis for the regulation of gene expression in eukaryotic cells (26) (see enclosed reprint). 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.

Previous studies have shown that in 3T3 cells (28,30) as well as other cell cultures (29,31), RNA does not turn over in growing cultures but does turn over in confluent cells. Subconfluent cells were labeled with ^{32}P and the label chased during RNA turnover when the cultures were confluent. During the chase, other cultures which were growing were labeled with ^{33}P . When the ^{33}P labeled cultures became confluent both the ^{32}P and ^{33}P labeled cultures were combined and their RNA extracted. Both the ^{32}P and ^{33}P labeled RNA had therefore been labeled under identical conditions. The only difference between them was that the ^{32}P labeled RNA had also had an opportunity to turn over.

If our hypothesis were correct, then segments of RNA resistant to ribonuclease should be found that should be conserved. Thus in vitro ribonuclease treatment of the above ^{32}P and ^{33}P dual labeled RNA might produce ribonuclease resistant oligonucleotides. Moreover, a higher

ratio of $^{32}\text{P}/^{33}\text{P}$ in these oligonucleotides in comparison to the rest of the RNA macromolecule might suggest evidence for conservation of these oligonucleotides. If the whole RNA molecule were turning over completely and homogeneously, then the $^{32}\text{P}/^{33}\text{P}$ ratio should be the same throughout the molecule. If segments of RNA were being reutilized during turnover then they would reincorporate the ^{32}P label. Examination of such molecules would then show enrichment of certain segments with ^{32}P , thus raising the $^{32}\text{P}/^{33}\text{P}$ ratio in these segments.

The dual labeled RNA was fractionated on sucrose gradients and the RNA fractions were digested with crude spleen phosphodiesterase, which in an unpurified form contains both exonucleolytic and endonucleolytic activity (32,33). Pretreatment of the RNA with alkaline phosphatase did not alter the results obtained. The $^{32}\text{P}/^{33}\text{P}$ ratios in both the ribonuclease resistant and ribonuclease sensitive portions of each RNA fraction are not significantly different along the gradient except for the 18s region where the ratio of radioactivity $^{32}\text{P}/^{33}\text{P}$ in the ribonuclease resistant material is about twice as large as that in the ribonuclease sensitive material.

These results suggest that the ribonuclease resistant RNA which sediments in the 18s region has a slower rate of turnover than the rest of the RNA sedimenting in the 18s region.

The 18s ribonuclease resistant material could represent either entire molecules or segments of 18s RNA molecules which are particularly resistant to ribonuclease. We have therefore examined the length of the molecules making up the ribonuclease resistant (rr) 18s RNA. The 18s-RNA, after phosphodiesterase treatment, was chromatographed on DEAE cellulose columns in the presence of 7 M urea. By using a NaCl gradient in urea, RNA oligonucleotides can be separated by chain length (34).

The column eluate showed a low broad peak eluting with about 0.3 M NaCl, as well as the early prominent mononucleotide peak and much lower dinucleotide peak. The radioactivity also peaked in the same region of the column eluate. 88% of the radioactivity placed on the column was recovered in the column eluate. No significant amount of either radioactivity or optical density could be eluted from the column by addition 3 M NaCl. The rr RNA from the 18s region of the gradient appears therefore to represent oligonucleotide segments of 18s RNA and not whole 18s molecules, since complete 18s molecules would not elute with only 0.4 M salt. Yeast t-RNA cannot be eluted with the salt gradient used but does elute with 3 M salt, indicating that this peak does not represent contamination with 4s RNA and that the rr-RNA represents RNA sedimenting slower than 4s.

To determine whether the 18s ribonuclease resistant oligonucleotides (rro) represented truly RNA and not contaminating DNA, both the 18s and 28s RNA, as well as the rro, were treated with alkali. After alkali treatment, no detectable radioactivity above background could be found in the PCA precipitates of pooled 18s or 28s RNA or in the rro of these species. The alkaline hydrolysis also completely eliminated the previously observed rro peak.

Experiments were also done to rule out the possibility that the rro represented a segment of poly A associated mRNA. The pooled 18s RNA was passed through an unmodified cellulose column prior to ribonuclease treatment to remove any poly A associated mRNA. This pretreatment had no significant effect on the height or position of the rro peak on the DEAE cellulose chromatogram. Although these experiments did not eliminate the possibility that a minor fraction of the rro was derived from mRNA it did suggest that the majority of the rro was probably derived from 18s ribosomal RNA.

The results suggest that regions within 18s and 28s RNA (presumably ribosomal in origin) exist which are relatively stable to ribonuclease. The results also suggest that such regions may be conserved during the synthesis of new RNA molecules.

The degradation of 28s has been reported to result in the production of minor species of RNA which might have s values close to the 18s RNA. To rule out the possibility that such 28s RNA contamination was responsible for our results, we have labeled cells when confluent with ^{32}P and then chased the label. These cells were then combined with growing cells labeled with ^{33}P . If our results had been the result of contamination by 28s breakdown products then no change in our results would be seen with this labeling scheme. If, however, reutilization of rro produced our results, then the $^{32}\text{P}/^{33}\text{P}$ ratio for the 18s rro would have been less or equal to the remainder of the 18s RNA and the 28s RNA since reutilization of rro at confluence would have prevented any significant ^{32}P labeling of the rro. Our results were consistent with the reutilization hypothesis and showed no differences in $^{32}\text{P}/^{33}\text{P}$ ratio in either the 18s rro or the remainder of the 18s RNA or the 28s RNA.

2) Ribonuclease Resistant Oligonucleotides

The RRO has been further characterized this year. The cause of RNA ribonuclease resistance has been attributed to methylation of nucleoside ribose (35-38), dual strandedness (39,40), or base composition (41).

Alkaline hydrolysis of RNA containing methylated ribose results in the production of dinucleotides because of the necessity for a free 2' OH group for alkali induced cleavage. However, alkaline hydrolysis of RRO produced only mononucleotides, indicating no significant methylation of RRO.

Dual strands of RNA can be separated by heating. The RRO were heated to 90°C for ten minutes and then plunged into an ice bath. The RRO however remained resistant to ribonuclease, suggesting that dual strandedness did not account for their ribonuclease resistance.

Dual stranded RNA has been shown to adhere more strongly to hydroxyapatite columns than single stranded RNA. RRO were adsorbed to hydroxyapatite. Whereas double stranded RNA is eluted predominantly with 0.24 M phosphate buffer, the RRO, like single stranded RNA, was removed with 0.12 M phosphate buffer. These results again suggest that RRO are not dual stranded.

We have also studied the base composition of RRO using the thin layer chromatographic system of Randerath (42) as described in the next report. The unlabeled RRO were isolated and then labeled in vitro with tritium borohydride. Our results show a very high proportion of guanosine and cytosine (>85%) with a much smaller amount of adenosine and uridine. The ribonuclease resistance of RRO possibly is the result of the high content of these two nucleosides.

We have also sought to determine the length of the RRO. We elute our DEAE cellulose urea column with 0-0.4 M NaCl. A mixture of t-RNA does not elute under these conditions, indicating that RRO are smaller than the 80 nucleotides of t-RNA. To further define the average length of RRO, we have been using the procedures described in the next report of in vitro labeling. First, we label the end group and after hydrolysis, we label all the nucleosides. The ratio of end label to total nucleoside label should give us the average length of RRO.

Some difficulties have been encountered because the standard hydrolysis procedure has made use of enzymes. The RRO has shown significant resistance to the hydrolysis by these enzymes. We have

therefore turned to alkaline hydrolysis which does go to completion. Alkaline hydrolysis, however, results in a considerable amount of salt which must be eliminated before thin layer chromatography. The charcoal technique described in the next report has been successful in removing the salt and in vitro labeling and thin layer chromatography can now be applied to determining the average length of RRO.

3) Reutilization of Oligonucleotides

Our hypothesis for the regulation of gene expression (26), discussed earlier, suggests that segments of RNA from breakdown of high molecular weight RNA are reutilized en bloc in the synthesis of new RNA. We have, during the past year, sought direct evidence for this feature of the hypothesis. We have added radioactively labeled small molecular weight RNA to cells and nuclei and looked for radioactivity in high molecular weight RNA as evidence for en bloc reutilization.

Our experiments have principally concentrated on nuclei for three reasons. First, nuclear transcription, free of cytoplasmic influence, is a more basic system with fewer variables. Secondly, the problem of breakdown of small molecular weight RNA to mononucleotides, and reutilization of mononucleotides rather than oligonucleotides, is eliminated. Nuclei require nucleoside triphosphates and therefore label within mononucleotide degradation products is not reutilized. Thirdly, we anticipate difficulty in getting large amounts of added RNA to penetrate the cell membrane and relatively little difficulty in getting RNA to penetrate the nuclear membrane.

Small molecular weight RNA was produced by endogenous cellular nucleases. Cells were frozen and then incubated at 37°C for 30 minutes three times, then lysed in hypotonic buffer and again incubated at 37°C. These treatments activated endogenous ribonucleases resulting in

significant digestion of high molecular weight RNA to low molecular weight fragments. Such cells were phenol extracted. The aqueous layer from the last extraction was extracted with ether and then dialyzed. The volume of the aqueous layer was reduced and the aqueous layer was put on 5-30% sucrose gradients. The first seven fractions containing all RNA less than about 7s in size represented about 95% of all the RNA on the gradient. These first 7 fractions were pooled and added to nuclear preparations.

The nuclear preparations used were from mouse myeloma cells (43), Hela cells (45), 3T6 cells (44) and chick erythrocytes (22). The chick erythrocyte nuclei used degraded RNA from whole chick embryos. In none of these systems was there significant incorporation above the background of the added degraded RNA. This prompted a restudy of these nuclear transcription systems. A significant drawback of all of the above nuclear transcription systems is that they all may merely be adding nucleotides to pre-existing RNA strands rather than initiating new RNA synthesis. If our hypothesis is correct that degraded RNA serves as primer for RNA synthesis, then the lack of significant incorporation in our experiments may be due to a fundamental limitation of the nuclear transcription systems---their inability to initiate transcription.

A further difficulty of these nuclear transcription systems was their large amount of incorporation of a labeled nucleoside, uridine (about 25% of the nucleoside triphosphate level). Although these systems are supposed to incorporate only nucleoside triphosphates, they apparently have significant cytoplasmic activity to triphosphorylate exogenously added nucleoside. This means that we cannot use these systems, as we earlier supposed, to preclude incorporation of labeled mononucleotides.

A third objection to the nuclear transcription system also has arisen, as a result of our experiments. All the systems we studied showed only low levels of incorporation which lasts, at the most, about 30-40 minutes. This limited our ability to detect the small amount of incorporated label that we expected to find.

For these limitations of nuclear transcription systems, we have now shifted our attention to whole cell incorporation of degraded low molecular weight RNA. Our procedures are outlined in the Renewal Proposal.

B. Radiation Effects on RNA

Whenever ionizing radiation is employed in experimental, diagnostic or therapeutic programs, it is necessary to determine the dose of radiation that is actually being absorbed by the exposed biological materials. In the event of nuclear accidents which are remotely possible at nuclear power stations and of great public concern, a rapid and relatively exact knowledge of dose exposures is required for proper treatment. At the present time, many sophisticated systems are available to provide dosimetry measurements which in addition to direct dosage, can account for radiation backscatter, surface and/or skin contours and tissue inhomogeneity. Many textbooks and articles are available which describe these systems in detail (46 - 51). In conjunction with computers and various types of simulation systems, the available physical and chemical systems (47, 52-54) do provide relatively accurate estimates of absorbed doses in various biological tissues and materials.

The problem that arises in all the above mentioned systems is that the best they can do is estimate the dose that a given tissue will receive after taking into account many variations of the physical parameters. No exact method has as yet been established to determine absorbed dose in any biological system. In addition, many types of physical and chemical monitors are necessary to cover the wide spectrum of ionizing radiations ranging from low voltage X-rays to heavy, accelerated particles (55).

It should be noted that the great interest in the development of biological dosimeters has led to many novel and innovative systems. Sharsgard (56) has developed one such system whereby mammalian cells are implanted in gelatinous (tissue equivalent) material and are subsequently irradiated. The "gel" is then sliced and assayed for

colony survival of the cells. In this way "phantoms" of this material can be built to any size or shape and the cells embedded in it serve to provide an estimate of the absorbed biological dose.

Nucleic acid strand breaks are known to be a result of the action of radiation on cells or tissues (57 - 60). Since DNA has, to this time, been considered a primary target of cellular radiation damage (61-64) most studies have looked for correlations between known cellular survival phenomena and DNA strand breaks (64). Studies have also determined that DNA strand breaks result from both the direct action of radiation (10-20%) (65 - 67) and that the vast majority of the breaks (80-90%) are repaired within minutes after exposure (60,68,69). The number of strand breaks found have been shown to vary with tissue oxygenation (57), high temperature (60), and other variables (60,69,70) following the same general trends that are observed for cellular survival. No evidence has been found that radiation induced RNA strand breaks are repairable.

In a different field of research, a great deal of effort has been put forth to determine the actual structural components of the various types of RNA (71-78). A system has been developed whereby any free 3' terminal hydroxyl group on a ribose may, by a series of reactions, be labeled with a tritium (H^3) atom (75,76,77,78). This process has been employed in the analysis of the base structure of RNA (74,75,76,77) and may be used as well in the determination of RNA molecular weights (79) by calculating the ratio of end to total nucleoside labeling. Recently, a modification of this process has been used to detect a break in an RNA strand which was first thought to be a single strand.

We have been using, during the past year, the in vitro biochemical method of 3' hydroxyl labeling described above to assay for the radiation induced strand breakage in RNA. Almost all radiation induced damage in

RNA can be converted to a terminal 3' OH configuration (61,66). Assuming that a direct relationship exists between the number of strand breaks produced in RNA and the absorbed dose, it should be possible to develop a mathematical formulation linking the number of strand breaks observed in cellular RNA and the dose of radiation which the cell has absorbed.

1) Radiation Induced Strand Breaks in RNA.

As calculated in the accompanying research proposal, one rad will yield 10^{10} strand breaks in ribosomal RNA in 10^7 cells. It is very obvious however that in the labeling procedure every 3' end group hydroxyl will also label. Since there are, as calculated, 10^7 strands/cells, this would result in 10^{14} end group labels. The 10^{10} strand break labels would not be detected under these conditions. In fact, doses of 10^3 to 10^4 rads would be required to double the background end group labeling. In order to lower the resolution dose, it is apparent that the original 3' terminals of the RNA strands must be blocked and prevented from labeling.

Experiments have been performed this year on tRNA, since it is easier to obtain pure samples of tRNA than larger ribosomal RNA. At very high doses (50K rad) our results indicate that the hypothesis regarding the necessity of heating to expose the strand breaks (i.e. by disrupting the hydrogen bonding) is valid. This suggests that prior to heating the only available reactive site may be only the 3' terminal hydroxyl. Thus, employing the labeling technique described above but substituting unlabeled KBH_4 , the terminal group can be reduced to a stable, unlabeled nucleoside trialcohol. At this point, the RNA can be heated and the strand breaks exposed and labeled. Even if some of the breaks are available for reaction with the unlabeled KBH_4 (i.e. not protected by hydrogen bonding), the majority of the breaks can still be labeled and subsequently detected since the background will be almost entirely eliminated.

A second modification of the Randerath procedure has received considerable attention this year. This was made necessary by our need to examine large quantities of RNA (relative to Randerath). We must find minimal numbers of strand breaks in large numbers of RNA molecules in order to be able to measure relatively low doses of radiation. A method has been used (personal communication-D. Schwartz) employing activated charcoal which can selectively bind and release nucleotides or nucleosides. This allows for the removal of nonspecific label and high molar salt concentrations from the t-RNA sample after treatment. The background reduction achieved in this method is approximately 80 fold and the removal of salt is effectively 100% while sample recovery is in excess of 95%. Prior to the use of this technique, we were limited in the amount of RNA that could be analyzed because of streaking produced on the thin layer chromatograms from the salt in the reaction mixture.

2) Radiation Induced Alterations in RNA Nucleotides

In the above work the final analysis of mononucleosides is carried out by 2 dimensional thin layer chromatography. Certain anomolous results this past year led to a study on the effects of ionizing radiation on a mixture of ribonucleosides. It has previously been reported that base modifications occur when mononucleosides are irradiated (58,108) but the alteration in thin layer mobilities has not been investigated.

Employing the four major RNA nucleosides (G,C,A, and U) in aqueous solution, it has been found that dramatic changes occur in the 2 dimensional chromatograms following irradiation to 60K rad. A distinct and unique modification is observed in adenosine; the single mapping spot is distinctly split into two clearly separate entities. One maps to the same location as adenosine, the other location indicates greater mobility, but the compound is as yet unidentified. Examinations of the chromatographic

patterns of the other three nucleosides following 60K rad indicates that many different compounds are produced from each nucleoside, since, instead of a clear, single spot, a streak containing many smaller spots is observed. Further analysis and identification is in progress at this time.

C. Effect of Vasopressin on Radiation Sensitivity

Recent studies with animals indicate that vasopressin may be therapeutically useful in the protection of the gastrointestinal (GI) system from radiation damage during radiotherapy of adjoining regions (96). These studies assume that the principal action of vasopressin is to reduce blood flow in the GI system thereby lowering tissue oxygen levels. It is well known in radiobiology that the absence of oxygen or its depression to below 20 $\mu\text{M}/\text{l}$ in the cellular environment will protect cells from radiation damage by a factor of approximately 3 (32). Since it is difficult, in practice, to measure the oxygen level in the GI tract, all calculations of $[\text{O}_2]$ are based on percent reduction in blood flow. With the inherent inaccuracies in this method, it is possible that the vasopressin itself has some cellular radioprotective effect. To test the effect of vasopressin on radiosensitivity of V79 cells in culture, we have incubated varying concentrations of vasopressin and monitored cell survival after radiation by colony growth and compared this to cells irradiated in the absence of vasopressin. Assuming a blood content of 5 l for the human body, the average drug concentration in the whole blood was 10^{-3} units/ml (1X) of vasopressin. Therefore, we have used in the cellular medium 10^{-3} units/ml (1X) of vasopressin. Toxicity and plating efficiency studies were performed at drug concentrations of 100X, 10X, 1X, .1X and .01X for 6 hour exposures. The vasopressin was found to be entirely without deleterious effects over the time and concentration ranges studied.

Complete radiation survival curves have been obtained for control and drug treated populations. The drug concentration employed was 1X. No effect on cellular radiosensitivity was found. In the animal studies, there was continuous perfusion of the drug during radiation; it is possible, therefore, that the actual cellular accumulated concentration of the drug was much higher than 1X.

D. 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 RNA.

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 proteins amino acid residues (phenylalanine, tyrosine, tryptophan, histidine) seems to be a particularly viable possibility. Many studies (80-97) have been made using a variety of model systems - aromatic amino acids and dipeptides with nucleosides and dinucleoside phosphates; aromatic amines, aromatic amino acids, or peptides and peptide amides containing aromatic residues with DNA and polynucleotides; tryptophan and indole derivatives with the

purine and pyrimidine bases, nucleosides and nucleotides. 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 downfield shift of the hydrogen-bonded 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) effect of each molecule on the other (86). If the exchange is fast between free and complexed molecules on the NMR time scale--the expected case for a weak complex--only one, narrow, resonance peak will be observed for each individual proton in the equilibrium mixture. Slower exchange leads to somewhat broadened peaks; very slow exchange produces separate peaks for protons

in free and uncomplexed molecules. The observed frequency of the narrow peaks will be a weighted average of the resonance frequencies in the free and bound states (91). 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 (92). 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 (92).

Experimental studies on aqueous solutions of aromatic amines or amino acids with nucleosides or nucleotides clearly indicate that complex formation leads to upfield shifts, but no broadening, of the proton magnetic resonances associated with the aromatic ring protons of both components (80,90,93, 86,94). Héline et al. (93) 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-1¹, which is closest to the purine or pyrimidine ring. A decrease in the coupling constant between the H-1¹ and H-2¹ protons of the ribose (93,94) 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 (94).

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 (80). The observed chemical shifts also indicate that the aromatic rings are stacked parallel to each other in the complex (90).

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 Lawaczek (94) 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 (86). 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) to 9 M^{-1} for a tryptamine-5'GMP complex (pD 7.9, 37°C).

The largest upfield shifts of the heterocyclic base protons in such complexes are induced by tryptophan and related indole derivative. 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 (98-101) have calculated ring current and shielding values for aromatic amino acid sidechains and nucleoside bases. They calculated decreasing shielding values in the order tryptophan > phenylalanine > tyrosine > histidine, with tryptophan

exhibiting considerably larger shielding values than any of the others. They calculated that the intermolecular shielding due to ring current magnetic anisotropy of the bases decreases 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 (94).

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 (102). 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. 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 (9,18). 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. (93) 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 (88). 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 (103). Insertion of the aromatic side chains between the bases of DNA or polyA 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 (85). 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 side chains (100). Gabbay et al. (87, 102) 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 $-NH_3^+$ 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 (87). Tryptophan-containing peptides, perhaps because of tryptophan's stronger interaction with nucleic acid bases, show upfield chemical shifts which are independent of sequence (87, 102).

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 (104) 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 (102) (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 phenylalanyl residue and the base pairs. Observed chemical shifts are also dependent on the relative concentrations of peptide and nucleic acid, and on temperature (91).

Direct binding studies of dipeptide amides containing one lysyl residue and one aromatic amino acid residue with DNA by Gabbay et al. (102) 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. (105) 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.

We have taken two approaches to the examination of one possible mode of protein-nucleic acid interaction: ring stacking between the purine and pyrimidine bases of nucleic acids and the aromatic residues of proteins. One study concerns itself specifically with the synthesis and spectroscopic

analysis of a nucleotide-amino acid covalently bonded model compound, adenylyl-5'-(P → N)-L-tryptophan methyl ester. In such a molecule, assuming sufficient flexibility so that the rings can stack, it is not necessary to rely on complex formation for the observation of ring stacking. Thus, this model can provide parameters for less extensively stacked (intermolecular) systems. "Model-building" using molecular models, suggests that this molecule can assume conformation with extensive stacking. Our other approach (see below) involves the study of complex formation between L-tryptophan and its analogs with mono and dinucleoside phosphates under a variety of solvent conditions.

1. Synthesis and Spectroscopic Analysis of Nucleotide-Amino Acid Covalently Bonded Model Compound

The model compound, Adenylyl-5'-(P → N)-L-tryptophan methyl ester, was synthesized using a modified carbodiimide procedure described by Ohtsuka and coworkers (106), and subsequently used by Shaborova et al. (107) to synthesize adenylyl-5'-(P → N)-phenylalanine and adenylyl-5'-(P → N)-p-anisidine. Several modifications were made in the synthetic and purification steps: 1) Reflux was maintained at 82.5°C for four hours. 2) The progress of the reaction was monitored every fifteen minutes by thin layer chromatography on silica gel plates. These were then developed in a 7:2:1 by volume isopropanol: deionized water: concentrated ammonium hydroxide solvent system. 3) The reaction mixture was concentrated to a gum on a rotary evaporator and then dissolved in 50% aqueous pyridine. This solution was slurried over night with the pyridinium form of cation exchange resin (AG 50 W-X) to remove carboxyamidine. 4) The aqueous pyridine solution was rendered anhydrous by repeated additions of dry pyridine followed by drying on the rotary evaporator.

As in the earlier work, initial purification of the compound was accomplished by extraction with n-hexane. Cyclohexylurea was removed by

filtration. This was followed by extraction with a 2:1 by volume ether:n-hexane mixture. After suction filtration the precipitate was washed three times with ether. Our major departure from earlier work is that all further purification was effected by means of liquid chromatography.

A Dupont 420 Liquid Chromatograph was fitted with a 0.5 analytical strong anion exchange column for the initial separation pilot. Satisfactory separation, that is symmetrical Gaussian peaks, was achieved on this column using a degassed 7:2:1 by volume solvent system of isopropanol: water: concentrated ammonium hydroxide (same solvent system as for the TLC). Subsequently, it was desired to scale this analytical pilot up to preparative dimensions so as to effect the purification of a maximum amount of product. A 0.5 meter preparative strong anion exchange column was obtained from DuPont for this purpose. Adequate preparative separation was never realized, however, with the 7:2:1 solvent system, due to a chemical modification the manufacturers had made in the solid phase of the column. Consequently, further purification of the compound was completed on the 0.5 meter analytical column operating in an overload condition. Product saturated in isopropanol was introduced to the system in 3 microliter injections. At least twenty injections were required to obtain enough product for spectroscopic analysis.

Identification of the produce as adenylyl-5'-(P $\rightarrow$ N)-L-tryptophan methyl ester was then attempted. Thin layer chromatography of the product after separation and subsequent drying on the rotovac showed a single spot. The spot appeared at an R_f value intermediate to tryptophan methyl ester, and adenosine monophosphate, and corresponding to that obtained by Shabarova et al. for the material they identified as adenylyl-5'-(P $\rightarrow$ N)-L-tryptophan methyl ester. Both Ultraviolet and Fourier Transform NMR studies were made on the purified product. Spectral studies were done for identification as well as for our primary purpose, the investigation of ring stacking.

Ultraviolet spectra were recorded on a Perkin-Elmer 202 Ultraviolet-Visible Spectrophotometer. Proton Nuclear Magnetic Resonance spectra were recorded on a Varian CFT-20 NMR Spectrometer, operating at 80MHz.

Ultraviolet spectra were obtained for all starting materials and for the purified form of the product at ambient temperature, $25 \pm 2^\circ\text{C}$. The maximum absorbance of AMP was found to occur at 260 millimicrons. Tryptophan methyl ester absorbed most strongly at 285 millimicrons. The purified product absorbed at a wavelength intermediate to these, 270 millimicrons. Now in progress is a comparison of the product UV spectrum with a direct sum of the spectra of AMP and tryptophan methyl ester. The UV spectra were also employed for concentration determination. The extinction coefficient for the synthesized product was estimated and used to determine product concentration.

Proton NMR spectra were obtained for all of the starting materials as well as the purified product. Variable temperature studies at concentrations on the order of 10^{-5} Molar product in D_2O were conducted. Ambient probe temperature was initially determined to be $37 \pm 1^\circ\text{C}$ using a copper-constantan thermocouple. Probe temperature was subsequently varied at ten degree intervals from about 0°C to 40°C and then from 35°C to 5°C . It should be noted that the spectral results obtained were reproducible both in terms of sample trials and the direction of the temperature change. Analysis of the spectra, now in progress, involves close attention to upfield shifts in the aromatic region as seen in the purified reaction product compared with the starting materials, tryptophan methyl ester and adenosine monophosphate. Upfield shifts would be predicted with ring stacking, and since at these dilute concentrations intermolecular interactions are at a minimum, ring stacking will be interpreted as being intramolecular. Therefore, these results infer the successful synthesis of a protein-nucleic acid model compound. Preliminary analysis reveals that upfield

shifts increased as the temperature decreased; with a lowering of the temperature line widths also broadened. Changes in coupling were also noted. Thin layer chromatography was run before and after each spectroscopic sequence to insure product integrity. Concentration of the sample was determined by UV measurements. Data obtained to date, TLC, UV, and NMR, indicate that adenylyl-5'(P→N)-L-tryptophan methyl ester has been synthesized and that stacking is occurring between the adenine and indole ring systems in this compound.

2. Intermolecular Interactions Between Tryptophan and Dinucleoside Phosphates

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. In our earlier work, the stoichiometry and geometry of the interaction of tryptophan with dinucleoside phosphates was investigated by monitoring upfield shifts in the proton nuclear magnetic resonance (NMR) 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 NMR 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.

NMR spectra were recorded on a continuous wave 100 MHz instrument, a Varian AA-100, at Harvard University, operating in the frequency sweep mode. Acquisition by this laboratory of a Varian CFT-20 adapted for protons, i.e. a fourier-transform 80 MHz instrument is allowing the gathering of 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.

Significant stacking was observed despite electrostatic repulsion between rings caused by ring proton under highly acidic solvent conditions. Interactions between like and unlike molecules were compared and we were able to propose geometries for the stacked complexes (80).

This past year we continued our study of intermolecular interactions. We sought tryptophan analogs, which were more soluble in neutral aqueous media, so that we could work at a pH closer to the physiological one of 7.4. Tryptophanamide hydrochloride and tryptamine hydrochloride could be dissolved at the desired concentration of 0.050 M in a deuterated buffer (pH 7). Deuterated buffer was required for NMR studies. KD_2PO_4 was prepared by equilibrating KH_2PO_4 with D_2O , separating the KD_2PO_4 - KH_2PO_4 mixture on a rotary evaporator and re-equilibrating in D_2O until an NMR spectrum of the solution showed a narrow HDO resonance. The buffer solution contained KD_2PO_4 and NaOD.

Preliminary NMR data of buffered solutions containing 0.050 M tryptophanamide hydrochloride or 0.050 M tryptamine hydrochloride with ApA, CpA, ApC and CpA showed significant upfield shifts of aromatic ring protons.

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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

*Reprints
Removed*

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

Kolodny, G.M., A New Hypothesis for the Regulation of Gene Expression by Activator RNA: In Extracellular Matrix Influences on Gene Expression", Ed. H.D. Slavkin and R.C. Greulich, p. 75, Academic Press, N.Y. (1975).

Kolodny, G.M., The Regulation of Gene Expression in Eukaryotic Cells, Medical Hypothesis 1, 15 (1975).

Kolodny, G.M., Turnover of Ribosomal RNA in Mouse Fibroblasts (3T3) in Culture, Exp. Cell Res. 91, 101 (1975).

Hetzel, F.W. and Kolodny, G.M., The Relationship between Repair of Potentially Lethal Damage and Radiation Induced Giant Cell Formation in 3T3 Cells, Radiation Res. 66, 1976.

Hetzel, F.W. and Kolodny, G.M., Alterations in RNA Turnover Following X-Irradiation, Radiation Res. 62, 585 (1975).

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., A Possible Role in Gene Regulation for Reutilized Segments of RNA from the same or different Cells. Proceedings of International Titisee Conference on "Information Transfer between Cells." April, 1975. Titisee/Black Forest, Germany.

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

Kolodny, N.H., Neville, A.C., Coleman, D.L., and Zamecnik, P.C., PMR Study of Interactions in Solution between Tryptophan and Dinucleoside Phosphates at low pD. J. American Chem. Soc.

Hetzel, F.W. and Kolodny, G.M., Radiation Induced Giant Cell Formation; The Influence of Potentially Lethal Repair

Hetzel, F.W. and Kolodny, G.M., Radiation Induced Alterations in RNA Turnover in Mammalian Cells.