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Nuclear Ribonucleic Acid in Regenerating Wound Tissue

Thomas Cherian Thachet

Loyola University Chicago

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NUCLEAR RIBONUCLEIC ACID IN REGENERATING WOUND TISSUE

by

THOMAS CHERIAN THACHET

A DISSERTATION

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF LOYOLA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

JUNE

1967
LIFE

Thomas C. Thachet was born in Neendoor, Kerala, India, on
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He wishes to thank his parents for their sustained inspiration and encouragement during the course of his education.

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ABSTRACT

Formation and deposition of collagen in the wound tissue of rats take place most vigorously about 7-10 days after wounding. The amount of RNA in the regenerating wound tissue also reaches the maximal level during the same period. At this stage of regeneration, The RNA in the cellular nuclei is formed especially rapidly. The nature of the relation between collagen formation and the RNA molecules involved, however, is unclear. The purpose of this investigation was to obtain further information about the nuclear RNA in wound tissue and its possible relationship to the synthesis of collagen.

Wound tissue at different stages of regeneration, obtained from rats, was homogenized and the nuclear material was isolated. The nuclear RNA was extracted from phenolic suspensions of the nuclear material and it was separated into several fractions on Ecteola columns by stepwise elutions with increasing concentrations of lithium chloride solution. The approximate molecular size and the purine and pyrimidine composition of the nuclear RNA fractions were determined.

The largest amount of nuclear RNA could be isolated when the total cellular RNA was greatest and collagen synthesis was found to be most extensive. Base composition of the nuclear RNA fractions, with the highest molecular weight (supposedly corresponding to ribosomal RNA),
was essentially the same at all the stages of regeneration studied. The concordance of base composition of these fractions was observed even though the type of cells found in the granulation tissue changes rapidly during the various phases of regeneration.

The fractions which were considered to consist largely of m-RNA had a different base composition when isolated from wound tissue with almost no fibroblasts (in the early stages of regeneration) and from wound tissue containing mostly fibroblasts (in the later stages of regeneration). A gradual decrease in the purine to pyrimidine ratio of these nuclear RNA fractions was noticed as the regeneration progressed.

The rate of formation and turnover of the nuclear RNA fractions of wound tissue were measured by following the uptake of tritiated uridine into these fractions. The labeling experiments were carried out on the 8th day of regeneration, when the fibroblastic proliferation and deposition of collagen in the wound tissue were thought to be high.

The ribosomal RNA is formed relatively slowly and accumulated in the nucleus. Evidence of slow turnover of r-RNA, presumably by the movement of it from the nucleus to the cytoplasm, was observed.

The m-RNA fractions were synthesized and turned over considerably more rapidly than either of the s-RNA or r-RNA fractions. One m-RNA fraction, representing about 5% of the total RNA isolated from the nucleus
was formed extremely rapidly - at least four times as fast as the next most rapidly formed m-RNA fraction. The rapid formation of RNA molecules in this fraction, at the time when rapid formation of collagen is taking place in the regenerating wound tissue, may be taken as presumptive evidence for a requirement of some m-RNA component of this fraction in the synthesis of this protein (collagen).
CHAPTER I

INTRODUCTION

The term "wound healing" refers to the reparative process of many tissues in response to disease or injury. For many centuries the process of wound healing had been studied extensively and carefully. Yet, to all intents and purposes, these early studies did more to confuse than to elucidate the mechanism of the processes involved. The earlier studies of wound healing were mainly morphological; later, mensuration of wound areas and tensile strength were also emphasized. More recently biochemical techniques have been employed to follow the compositional changes in the healing of wounds. The biochemical work on the metabolism of wound tissue and the apparently related connective tissue has been devoted largely to that of proteins (176). The function and metabolism of mucopolysaccharides, collagen, lipids, endocrine secretions and other substances affecting the multiple biochemical events taking place in the healing process have been reviewed in detail (24).

Regenerating wound tissue is actively involved in synthesizing many proteins, and particularly collagen. Collagen is the extra-cellular fibrous protein of connective tissue and is generally regarded as responsible for giving the healing wound its tensile strength. The synthesis of collagen and mucopolysaccharides has been attributed to the
fibroblasts (27). The cellular population of granulation tissue eventually becomes predominantly fibroblasts (24). In the early stages of healing there are only a few fibroblasts and the collagen content of the granulation tissue is quite low. But the collagen content of wound tissue begins to rise sharply at about the fifth day after wounding (69).

Nucleic acids are known to be involved in the biological mechanism of protein formation. The biosynthesis of the monomeric precursor molecules of collagen undoubtedly follow the mechanism for the synthesis of other proteins in the cells. This mechanism involves amino acid activation, formation of amino-acyl-s-RNA and transfer of the amino acid to the growing peptide chain on ribosomal RNA (r-RNA) as directed by messenger RNA (m-RNA). The nucleotide sequence of m-RNA is a reflection of the nucleotide composition of DNA and m-RNA is the agent which passes the word of heredity from gene to newly synthesized protein. Since collagen has a unique amino acid composition (84, 129) which is sharply divergent from other proteins, it might be expected that the particular m-RNA molecules involved in the synthesis of collagen should have a highly peculiar nucleotide composition and sequence.

Since DNA is essentially confined to the nucleus and RNA is produced on the DNA, it is evident that the m-RNA is synthesized in the nucleus and then transferred to the cytoplasm where protein synthesis is
taking place. The nucleolus has been suggested to be the principal storage depot of RNA in the nucleus of the cell (148). Incorporation studies with labeled RNA precursors in systems which are actively engaged in the synthesis of collagen reveal that the uptake of radioactivity is in the nucleus within the first hour. Later, the radioactivity appears in the cytoplasm.

The RNA content of wound tissue increases with time and reaches a maximal concentration in approximately 7 - 10 days after wounding (69, 32, 177). The incorporation and turnover of $P$ in the nuclear RNA of regenerating wound tissue parallel the deposition of collagen in the wound tissue (179). Moreover, nuclear RNA, isolated at different stages of wound tissue regeneration, shows a purine to pyrimidine ratio considerably lower than that of the RNA isolated from nuclei of other tissues (50, 63).

Cellular proteins as well as collagen are produced during wound tissue regeneration. So the nuclei isolated from the wound tissue may be expected to contain a variety of RNA molecules responsible for the production of various kinds of proteins. The nuclear RNA of normal cells has been separated into several fractions by use of modern techniques. If the nuclear RNA of wound tissue is fractionated, and the base composition
and the rate of formation of each fraction are determined, it may be possible to identify the RNA responsible for the formation of collagen.
CHAPTER II
COLLAGEN AND WOUND HEALING

All multicellular organisms contain a framework of solid material which, in part, maintains their shape. In plants this consists of long chain carbohydrates such as cellulose. In animals, materials of primarily carbohydrate origin are involved and may sometimes form a part of the structural material, as for example, chitin in invertebrates, but the dominant materials are proteins. Of these proteins, collagens appear to be the most important and widely distributed. They appear to be characteristic structural proteins of soft tissues of all animals, but are perhaps typically the dominant structural materials in vertebrates, in which they may constitute 20-35% of all the body proteins.

Collagen is a fibrous protein. The individual molecules are thread-like, and are arranged together in threads of higher orders of size to form a continuous framework throughout the body. This fibrous framework is closely associated with other materials filling spaces within it and cementing it together. Such materials include crystalline solids, as in bone, but generally they appear to be gels containing a high proportion of water, associated with mucopolysaccharides. The major component of the whole fibrous framework is collagen. Parenthetically, it might be pointed out that more is known about this than any other component of
connective tissues.

Collagen has unique chemical and physical properties. The most important feature from the point of view of molecular architecture is that collagen contains a large proportion (33%) of glycine (14, 129). The high proportion of this small amino acid allows the polypeptide chains of collagen to come closer to one another and form a structure of high mechanical strength. Collagen contains two unusual amino acids, hydroxyproline and hydroxylysine. The latter is found in relatively small amounts (usually less than 2%) but as much as 20% of the former may be found. In mammals these hydroxy amino acids appear to be confined exclusively to collagen. Other peculiarities in amino acid composition are also found in collagens. For example, tryptophane and cystine are absent and only small amounts of tyrosine and methionine are present (84, 129).

In the last decade, the structure of collagen has been extensively studied by X-ray crystallographic and other methods (140). The structure now generally accepted consists of three polypeptide chains coiled together in helical fashion like a three-stranded rope. The three chains are held together by hydrogen bonds between carbonyl and amino groups of glycine residues in adjacent peptide chains. Every third amino acid in the chain is glycine. About another 30% of the amino acid residues consist of proline plus hydroxyproline. Glycine has only a hydrogen atom in place of the side chains of the other amino acids, and the sites where it is
found form a sort of groove running spirally round the molecule. The above structure is supported by analysis of peptides formed by enzymic or acid hydrolysis of collagen (see Table I) (143, 169).

Investigation of collagen by electronmicroscopy and physico-chemical methods indicates that the individual molecules, of molecular weight of about 350,000, are relatively rigid rods about 2800Å long and 14Å in diameter (13). The name tropocollagen has been given to this molecule. Piez, et al. (129) have represented tropocollagen as containing two types of subunits, and . These are present in the proportion of two to one . These subunits are polypeptide chains of similar size, but of different amino acid composition (see Table I). Under certain conditions, dimers of and can be obtained from collagen. These are designated as and ; is a dimer consisting of a unit crosslinked to a unit, while is a dimer consisting of two units crosslinked in a similar fashion.

Tropocollagen molecules are asymmetric and associated together by their end regions, where the ends of the three polypeptide chains project at different lengths. Under suitable conditions tropocollagen molecules associate together spontaneously to form long fibrillar structures with the typical banded appearance of naturally occurring
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Tropo-collagen</th>
<th>l 1 Subunit</th>
<th>l 2 Subunit</th>
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<tr>
<td>3-Hydroxyproline</td>
<td>0.09</td>
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collagen. Under specific conditions, they can be caused to associate together in other ways to give the so-called fibrous long-spacing (FLS) collagen, with a repeat unit of 2800 Å instead of the normal 640-700 Å. Other ways of association give individual segments like cotton reels called segment long-spacing (SLS) collagen. These appear to be formed as follows: the 14 x 2800 Å asymmetrical tropocollagen particles associate together by their end regions, where the three chains of the helix do not end together but project by different lengths. These end regions have specific characteristics of amino acid composition and in particular contain tyrosine. By the interlinking of these free ends, the molecules join together end to end. If these ends are A and B, normal collagen is formed by association of molecules in parallel array all pointing in the same direction so that the whole fibril is polarized with different A and B ends and overlapping by a quarter of their length:

\[
\begin{align*}
A & \quad \text{BA} \quad B \\
A & \quad \text{BA} \quad B \\
A & \quad \text{BA} \quad B \\
\end{align*}
\]

FLS collagen is formed by association in antiparallel array without overlap:

\[
\begin{align*}
A & \quad \text{BA} \quad B \\
B & \quad \text{AB} \quad A \\
A & \quad \text{BA} \quad B \\
\end{align*}
\]
SLS collagen is formed by association in parallel array without overlap:

A \begin{array}{c}
A \\
A \\
A \\
A 
\end{array}
B \begin{array}{c}
B \\
B \\
B \\
B 
\end{array}

This last type of association can be brought about by precipitating collagen in the presence of ATP. ATP is supposed to produce this structure by reacting with the basic amino acid side chains aggregated at one end of the molecule (53).

Even though the above description represents the general features of the structure of collagen, evidence of further complexity is shown by a linkage between glycine and carbohydrate (83).

Collagen fibers are relatively inextensible and have a high tensile strength. In acid or alkaline media, collagen imbibes water markedly and swells. Certain forms of collagen can be resolved without denaturation. Most forms of it dissolve in hot water to form gelatin. It is a unique property of collagen which gives rise to its name. This process involves the irreversible destruction of the molecule by unwinding and separation of the helically coiled polypeptide chains. This effect is also produced by strong hydrogen-bond-breaking agents (28).

Soluble collagen represents a group of biologically young
collagen molecules which can be brought into solution under certain conditions without the loss of the native structure. This kind of collagen is considered to be newly formed. Neutral salt soluble collagen is thought to be a precursor of both acid soluble and insoluble collagens. The aging of collagen appears to decrease its solubility, but the exact nature of the change is not understood.

General information about the distribution of collagen in the animal body has been obtained from histological examination. Quantitative data are available only for animals. The larger amounts of collagen are found in those tissues which have primarily a mechanical function, namely, tendon, cartilage, bone, skin, and sclera of the eye (53). Complete analysis of whole mice have been performed and collagen formed about 25% of the total body protein. The skin contained about half of all collagen in the body (54).

During the growth in an individual, the collagen content of a tissue generally increases more rapidly than the weight, so that the concentration of the collagen increases (53). This may be partially accounted for as an adaption to the mechanical requirements of large size. It seems probable that a large part of the increase in concentration of collagen in fetal life can be accounted for by increase in fibril size, assuming the number of fibrils per unit volume to remain constant (61). Incorporation of labeled proline into collagen showed that collagen is turned over at a
relatively fast rate (161). This was followed by measuring the hydroxyproline excretion (161).

It is generally agreed that the fibroblast (and its variants, the osteoblast, chondroblast, etc.) are the cells responsible for the formation of collagen (75). There have been many histologic and electronmicroscopic studies about the formation of collagen in connective tissues, tissue culture and tissue explants (53). Schwann (144), who first described the connective tissue cell, believed that collagen fiber developed directly from the cell. Virchow (170) suggested that the cell secreted a soluble substance that became fibrillated outside the cell. More recent evidence supports this hypothesis and indicates that collagen fibers are formed largely outside the cell (53). Stearns (158) described cytoplasmic granules that secreted a soluble precursor that was transformed extracellularly into a fibrous form of collagen. This was confirmed by electronmicroscopic studies. Porter and Vanamee (131), studying the fibroblast tissue cultures, showed that the earliest fibers are found at the cell surface and appear to develop extracellularly by accretion of material from the extracellular nonfibrillar matrix. An important piece of evidence in favor of extracellular fibrillation has been provided by Robbins et al. (142). These authors found that fibrils failed to form in tissue cultures of fibroblasts grown in the presence of an anticollagen serum. Instead,
amorphous masses developed extracellularly, having the staining properties of collagen and devoid of a characteristic collagen structure under the electron microscope. These were apparently antigen-antibody complexes formed in the extracellular space before the soluble collagen precursor had time to aggregate to form the normal collagen fibril (53).

Collagen is the extracellular fibrous protein of connective tissues. In the process of wound healing it is generally regarded as responsible for giving tensile strength to the healing wound (53). Therefore, its mode of synthesis is particularly pertinent to a study of wound healing. If an area of skin down to the subcutaneous fascia is removed, some of the superficial tissue dies, and epithelium from the edges grows in over the surviving tissue in the floor of the wound which is usually filled with clotted blood (53). Simultaneously in the floor of the wound a growth process begins in which a cellular connective tissue is formed. Collagen is layed down in this new tissue, which is called granulation tissue. The cellular population of formed granulation tissue consists predominantly of fibroblasts, and other types of cells are also present. In the early stages of healing there are few fibroblasts and plasma contributes a number of cell types.

The origin of fibroblasts of the proliferating granulation tissue is not known. MacDonald (95) attempted to study the origin of these
fibroblasts and concluded from histological and autoradiographic methods, that they arose from fixed connective tissue cells adjacent to the adventitia of arteries and veins, around hair follicles and in the lining of muscle fibers in the subdermal tissue beneath and adjacent to the wounds. These conclusions were inferred from the time sequence of nuclear labeling from tritium-labeled thymidine and from the proximity of the cells. Fibroblasts were not observed to arise from lymphocytes, histocytes, polymorphonuclear leukocytes, fat cells, endothelial cells or fixed cells. Allgower and Hulligen (4) observed that the culture of the buffy coat layers from rabbit blood changed morphologically into fibroblasts. They concluded that mononuclear cells of blood are capable of changing into functional fibroblasts. Petrakis et al. (127) cultivated normal human leukocytes in millipore diffusion chambers which were implanted subcutaneously into autologous and homologous subjects. Histological observations were made over a period of six weeks. Mononuclear leukocytes differentiated into macrophages and polyblasts within a few days. At three weeks their morphological appearance was characteristic of histiocytes and fibroblasts and marked collagen formation was noted at 4 to 6 weeks.

The earliest signs of collagen formation in the wound are seen after 48 hours. It was detected histologically. Electron microscopic
investigations indicate that the first collagen formed in granulation tissue consists of thin fibrils resembling those first found in embryonic development (8). Later thicker fibrils, 600-800 A in diameter, are found which appear similar to the collagen fibers in other parts of the body. Examination of early granulation tissue broken up by ultrasound shows a high proportion of fibrils with tapered ends, as in early development, which indicates that the fibrils are shorter than normal (8).

The wounds are subjected to contraction, that is, to a reduction in wound area over and above that due to new tissue growth. The exact mechanism of this contraction is unknown. But many workers have attributed the process to tissue changes in the wound area rather than in the surrounding skin (53). This, in turn, has been associated with the collagen of the wound tissue. Another postulate has been that the wound contraction is due to the movement of the skin edge across the defect, presumably under the influence of forces in the skin (169).

Abercrombie et al. (2) studied collagen content of regenerating wound tissue in relation to contraction. Collagen formation and contraction proceeded concurrently up to 10 days. There was also a loss in tissue weight and the changes in the wound constituents might have exerted a contractile force. After 10 days, however, collagen synthesis
continued, but contraction had stopped. They also observed (1) that the wound area in scorbutic guinea pigs contracted even though collagen was not being formed. They proposed that the contraction was produced by the cellular population of connective tissues within the wound by rearrangement of the cells, packing them more closely together. Fibroblasts in tissue culture are known to have contractile properties (77).

Grillo et al. (45) also investigated tissue content in wound tissue in relation to contraction. The total weight of tissue, water content, collagen, hexosamine and tyrosine concentrations are measured. Contraction could not be correlated with changes in wound composition. However, they noted the presence of a rim of newly formed tissue attached to the advancing edge of the skin. Detachment of this skin rim resulted in retraction of the edge, and a possibility of a relation between active contraction and this marginal area was suspected. Further experiments (172) supported the hypothesis that this tissue in the wound margin was responsible for most of the contraction of the open wound. Removal of the granulation tissue from the middle of an open wound did not effect the rate or extent of contraction. If the wound edge was excised, there was an immediate distraction of the wound and it would result in a delay in contraction. The separations of the advancing skin margin from the wound base alone was sufficient to inhibit contraction. The results supported their hypothesis of the function of the tissue at the wound margin. Reynolds et al. (137)
also made a similar suggestion that a layer of "organizer cells" beneath the edge of an open wound was responsible for the initiation and control of contraction, migration of regenerate, fibrosis and epithelization.

There is some difference of opinion about the subsequent changes in the collagen content of wounds. Abercrombie and James (2), working with small wounds in rats, found a progressive increase in the total collagen content of the wound for up to 300 days. Dunphy and Udupa (33) followed wounds up to two weeks and recorded similar changes. Grillo et al. (45) have measured total tissue content and collagen. Although the proportion of the solids in the total wound tissue remained essentially constant, the distribution of components varied. No collagen was present in the first two days. A small but measurable amount of collagen was present in the wound on the third and fourth days. The total amount of collagen began to rise sharply at five days to a maximum at eight days. Then the amount fell initially rapidly up to ten days, and then more slowly. Collagen represented a smaller proportion of the total solids present when total wound content was maximum than it did later when total content fell. Concentration of collagen rose rapidly from the fifth day to about the end of the second week, after which it gradually approached the concentration found in the normal skin.

Hosoda (69) measured the hydroxyproline content in granulation
tissue of healing wounds. He found that the total amount gradually increased up to the tenth day, and the twelfth day. Neutral salt soluble, acid soluble and insoluble collagen fractions are extracted and the concentration of each fraction was studied during the wound tissue regeneration. The results suggest that an active synthesis of neutral salt soluble fraction and its rapid polymerization into insoluble collagen occurs on the 8th and 10th day in granulation tissue.

Guschlbauer and Williamson (51) studied the change in levels of proteins in the regenerating tissue. They determined the concentrations of tyrosine and hydroxyproline as a measure of cellular proteins (metabolically active) and collagen (metabolically relatively stable) respectively. The results on the rate of formation and deposition of collagen are in agreement with the observations of Hosoda (69). Collagen and hydroxyproline containing precursors are present only to a small extent during the early stages of tissue regeneration. Appreciable deposition of collagen appears after 7 days of wound repair. The observations on the cellular proteins does not change significantly during the course of the observed regeneration period. This was in agreement with the findings of Woessner et al. (181) that there is a constant level of soluble proteins during the formation of connective tissue in polyvinyl sponge implants.
CHAPTER III
NUCLEIC ACIDS AND PROTEIN SYNTHESIS

Several theories have been proposed to explain the mechanism by which amino acids are assembled to form the particular sequence which is characteristic of specific proteins (17, 90, 117). These theories proposed the existence of "templates" which are composed of nucleic acids and which direct chemical interaction of these templates with the amino acids so that the amino acid sequence has a specific order in the protein. More recently an alternative hypothesis had been put forward which differs in principle and detail from the theories mentioned above. According to this second scheme there are three distinctly different kinds of RNA molecules, soluble or transfer RNA (s- or t-RNA), ribosomal RNA (r-RNA) and messenger RNA (m-RNA). These RNA molecules are involved physically in protein biosynthesis.

Soluble RNA (s-RNA)

Soluble RNA molecules are polynucleotides estimated to contain about 50 nucleotide residues and to have a molecular weight of about 30,000 (3). The sedimentation coefficient of these molecules is calculated to be 3.5-4.5 Svedberg (S) units. These molecules are found in the cytoplasm of the cell and can be isolated from the subcellular particulate components. Various attempts to isolate the s-RNA molecules have
showed that there is a specific s-RNA for each individual amino acid. These RNA molecules differ from other cellular RNA molecules in that they are soluble in 1 M NaCl. The amino-acid-attaching end of the polynucleotide chain consists of two cytidylic acid residue at the terminus. Attachment of the amino acid to the RNA is possible only when these three residues are intact (59). The terminal adenylic acid residue is attached via its 5' phosphate group to the 3' position of the proceeding cytidylic acid residue. The activated amino acid becomes attached to the s-RNA through the 2' or 3' position of the ribose of the adenine nucleotide (22).

**Ribosomal RNA (r-RNA)**

Ribosomal RNA and its functions are probably the least understood of the various RNA species. It is estimated to comprise 80-90% of the total cellular RNA. It exists as ribonucleoprotein particles in which the protein contributes about 50% of the total weight (105, 162). The intact ribosomal particle is composed of two subunits having sedimentation constants of 50 S and 30 S, respectively (162). The RNA in the 50 S subunit has a molecular weight of 1,120,000 and sedimentation coefficient of 23 S. It is also possible that this ribosomal particle contains two RNA molecules, each having a molecular weight of about 560,000. The 30 S subunit of the ribosomal particle contains one of the smaller size RNA (155).
It appears that the RNA in the ribosomes consists of long continuous polynucleotide chains made up of 1500 to 4500 nucleotide residues each.

Several reports indicate that the active cellular component in protein synthesis consists of ribosomal clusters (polyribosomes) held together by a single m-RNA molecule. Evidence has been accumulated by electron microscopy and gradient centrifugation for the existence of polyribosomal aggregates in reticulocytes (48,49), rat liver (51,53), tissue cultured cells (123,163), bacteria (80), plants (112), chick embryo (85), sea urchin eggs (71), and slime molds (128).

**Messenger RNA (m-RNA)**

Deoxyribonucleic acid (DNA) provides the necessary genetic information for the synthesis of protein in the cell. For the most part, DNA is situated in the nucleus of the cell while most of the protein synthesis takes place in the cytoplasm. It seems unlikely that free amino acid could react directly and specifically with DNA to produce an ordered polypeptide sequence. Moreover, protein (hemoglobin) synthesis in mammalian reticulocytes, which lack nuclei, shows that DNA has no direct role in protein synthesis (89).

The formation of RNA in the nucleus and the recognition of its important role in protein synthesis suggested that the genetic information of nuclear DNA is somehow transmitted to an RNA which functions at the
sites of protein synthesis. Soluble RNA (s-RNA) is relatively small, 70 nucleotides, and it could not transmit the information required for the synthesis of proteins containing as many as several hundred amino acid residues. Moreover, s-RNA is specific for reacting with single amino acids. Therefore s-RNA can be excluded from the role as a carrier of genetic information from DNA. There is a considerable variation in the ratio of (G+C) / (A+T) (see Appendix III) in microbiol DNA (12). The base composition of r-RNA is remarkably uniform among different species of bacteria (108). Furthermore, in contrast to the wide variation in molecular weight of proteins in a single bacteria, its r-RNA shows a remarkably uniform distribution of molecular size (87). It is therefore unlikely, although not impossible, that r-RNA contains genetic information transmitted from DNA.

In 1961, Jacob and Monod (76) suggested the transient existence of a new RNA, which they called "the messenger" or m-RNA. Such a RNA could represent no more than a small percentage of the total RNA of the cell. They also predicted that this m-RNA would be somewhat unstable and is not accumulated in the cell, in contrast to the relatively constant presence of DNA, s-RNA and rRNA. The control of protein formation may be determined by the rate of synthesis of this template m-RNA (76).
The occurrence of a minor RNA fraction with a very high rate of synthesis in normal and phage-infected bacteria was demonstrated by exposing the bacteria for a short time to $^32$P and fractionating the RNA by gradient sedimentation (17, 47). The radioactive peaks which were isolated did not coincide with the ultraviolet absorption peaks of r-RNA and t-RNA. This suggested that they represented a different entity. The RNA fractions could be digested with ribonuclease and dilute bases.

Four nucleotides (A, C, G, and U) were recovered from the hydrolysis and the estimated base composition was found to be similar to that of homologous DNA (with uracil replacing the thymine of DNA) (46). In phage infected bacteria the DNA-type RNA was shown to form "hybrid" double helix specifically with heat-denatured single stranded DNA (52, 120). Such a "DNA-RNA hybrid" was demonstrated to occur naturally in bacteria (52). The hybrid double helix is characterized by its resistance to deoxyribonuclease and ribonuclease (52). These observations suggest that the DNA-type RNA has a base sequence similar and complementary (having bases paired with that of DNA by hydrogen bonding) to homologous DNA serving as the template for protein synthesis.

The m-RNA molecules of E. Coli are heterogeneous in size and have molecular weights in the range from 200,000 to 500,000 or higher (46, 47). Sedimentation analysis of an m-RNA preparation showed
sedimentation coefficients of 8-14 S. A freshly prepared m-RNA showed a wide range of distribution in sucrose density gradient analysis, suggesting that the smaller components were degradation products (58, 153). Later m-RNA of normal and phage-infected E. Coli could be fractionated on methylated bovine serum albumin (MBSA) column into four components (73, 160). All four compounds showed a DNA-type base composition. No precursor product relationship among the four components was noticed (73).

Whether the m-RNA carries the genetic information for protein synthesis can be tested by measuring the activity of m-RNA in stimulation of amino acid incorporation into proteins in a cell free system in which DNA and preexisting RNA are eliminated. Thus the four fractions of m-RNA molecules isolated from liver nuclei possessed messenger activity in varying specific activity (156). More recently one out of four RNA fractions isolated from rat brain nuclei and fractionated on methylated serum albumin column showed considerably increased messenger activity when added to a cell free system (62).

The enzymatic synthesis of RNA depends on the presence of DNA primer. This was accomplished by using enzyme preparations obtained from mammalian and plant nuclei (38, 70, 173) and bacterial extracts (18, 21). The enzyme catalysed the polymerization reaction
of the four ribonucleoside triphosphates, ATP, GTP, CTP, and UTP. The reaction was completely dependent on the presence of DNA. If DNA was omitted or treated first with deoxyribonuclease, no reaction occurred. The synthesized RNA had normal 3', 5'-phosphodiester linkages. The base composition reflected that of the added DNA. This suggested that the nucleotide incorporation was dependent on the ability of the bases of the ribonucleotides to pair with the bases in the primer DNA, by a mechanism similar to that for DNA duplication.

Since DNA acts as the template for RNA formation and nuclear RNA is formed very rapidly (36), the most logical place to search for m-RNA is in the cellular nuclei. Nuclear RNA is heterogeneous and an appreciable portion of it is contained in the nuclear ribosomes (130). The nuclei from which the ribosomes and the soluble fractions are removed may consist largely of chromosomes and nucleoli. RNA contained in the residual material has much higher rate of isotopic labeling than nuclear r-RNA (149, 183).

RNA can be extracted from cells, nuclei or nuclear residue by shaking them with aqueous phenol (28). RNA released into aqueous phase shows a low rate of isotopic labeling. Its base composition is always close to that of r-RNA. It can be resolved into r-RNA and s-RNA by chromatography or sedimentation analysis. The release of RNA by phenol into aqueous phase is not quantitative. Some RNA is left in the interphase
gel which is formed between aqueous and phenol phase. This RNA fraction (interphase RNA), obtained from the whole cells or nuclei, has a high rate of incorporation of its precursors (149, 183). Interphase RNA obtained from nucleolar residue of calf thymus contained a component with a base composition quite similar to that of DNA, and was tentatively identified as m-RNA (148). In these experiments, sizable quantity of DNA-type RNA from mammalian cellular material was isolated for the first time. This observation established the occurrence of DNA-type RNA in mammalian cells. The presence of m-RNA was also demonstrated in rat liver (64), leucocytes (101), ascites cells (109), and in regenerating rat liver (64).

Studies have been conducted to find out the distribution of DNA-type RNA (m-RNA) in the cell. Although it originates in the nucleus, it has to get into the cytoplasm to direct the protein synthesis. The whole cytoplasmic fraction and its subfractions yielded interphase RNA on phenol treatment. It was labeled at slightly higher rates than those exhibited by the bulk of RNA recovered from aqueous phase (81, 134, 149). A careful study did not reveal any significant difference in base composition between RNA of interphase and aqueous phase obtained from cytoplasmic subfractions (106). These results indicate the presence of only small amounts of m-RNA which may be transferred to nucleoli (139, 143). This
would suggest that m-RNA may be first synthesized on the chromosomes under the control of DNA and then transferred to and stored in the nucleoli. The RNA in the nucleoli may then be transferred to the cytoplasm (126).

Many workers feel that m-RNA is quite unstable (150). The general impression is that m-RNA may be preferentially degraded by ribonuclease after the disruption of the cell. When the concept of m-RNA was proposed, the idea was expressed that it could function only once as a template for protein synthesis and it would be destroyed every time polypeptide synthesis reaction was completed using its amino acid code (76). Such a mechanism would pose a limitation to the mode of s-RNA and m-RNA interaction. No proposed mechanism for protein synthesis has ever implied such a limitation. Moreover, a recent observation revealed that m-RNA molecule could produce at least several polypeptide molecules when its synthesis was completely blocked by actinomycin (72, 91, 138). Furthermore, reticulocytes which do not contain template DNA can synthesize quite an appreciable amount of hemoglobin (86). That m-RNA in normal cells is degraded continuously under in vivo conditions has not yet been directly proven. The label in m-RNA fraction was found to reappear in r-RNA and s-RNA (46). This may be
due to a complete depolymerization of m-RNA followed by an economical recycling of degradation products for the synthesis of another m-RNA, r-RNA and s-RNA. A more or less direct transformation of m-RNA into r-RNA or s-RNA is unlikely because of the difference in base composition. Moreover actinomycin D stops the synthesis of all the RNA classes almost immediately (72, 91, 138). This leaves little doubt in ruling out the possibility of a direct precursor product relationship between m-RNA and r-RNA.

The question of the molecular weight of m-RNA had been the subject of much discussion until it was recognized that m-RNA is heterogeneous with respect to sedimentation behavior, and hence presumably covers a wide spectrum of molecular weights. Knowledge of the range of molecular weights of m-RNA molecules appears of little value unless the molecular weight of the message could be related to the size of the peptide chain which it encoded. A direct approach to this problem was suggested by recent experiments (48, 49, 51, 53, 71, 80, 85, 112, 123, 163). According to these findings, the functional units of protein synthesis are ribosomal aggregates (ergosomes) consisting of closely spaced 70-80 S particles strung together by one continuous strand of m-RNA (48, 49, 51, 53, 71, 80, 85, 112, 123, 163). The metabolic turnover rate of this polyribosomal m-RNA is more rapid than that of
its structural counter-part, r-RNA. The m-RNA in this particle sediments in gradients between s-RNA and r-RNA (31, 100).

MECHANISM OF PROTEIN SYNTHESIS

The biological activity and structural uniqueness of proteins depend on the configuration assumed by the polypeptide chain. The molecular configuration, in turn, is mainly a function of the sequence of amino acids along the polypeptide chain. The mechanism of protein synthesis therefore must provide for the formation of the specific amino acid sequences and the specific three dimensional configuration of the polypeptide backbone. The working hypothesis for protein synthesis involves: a) the combination of each amino acid with its appropriate transfer RNA; b) transfer of amino acids to the peptide chain; and c) the transfer of information for specifying amino acid sequences from the DNA polynucleotide to the ribonucleoprotein particle (30, 66).

The formation of aminoacyl-s-RNA represents the first step in the protein synthesis. It is at this stage that discrimination among amino acids occurs. The formation of aminoacyl compounds takes place as a result of two intermediate reactions (68), namely the formation of aminoacyl-adenylate-enzyme complex and the transfer of aminoacyl moiety to the RNA molecules.
ATP + Amino Acid + Enzyme → Enzyme-(AMP-Amino Acid) + PPI
Enzyme-(AMP-Amino Acid) + s-RNA → s-RNA-Amino Acid + AMP + Enzyme.

This may be summed up to a single equation as follows:

ATP + Amino Acid + s-RNA → s-RNA-Amino Acid + AMP + PPI

The reactions are mediated by a single enzyme. A specific enzyme is required for the formation of each amino-acyl-s-RNA derivative. Each amino acid is linked to a separate and specific RNA chain through an ester bond. There appears to be more than one s-RNA which can transfer the same amino acid. The ribose portion of the terminal adenosine residue forms the ester bond with the carboxyl group of the amino acid.

The second step in protein synthesis is the transfer and the association of aminoacyl polynucleotide derivatives to the template-bound ribosomes. The transfer is made to the specific positions of the ribosomes, determined by the nucleotide sequence of the m-RNA. The aminoacyl-s-RNA is specifically aligned in relation to the growing peptide chain. The C-terminal ends of the amino acids are linked to the s-RNA. The amino groups of the incoming aminoacyl-s-RNA make a nucleophilic attack on the carboxyl carbon atoms of the peptidyl-s-RNA. This results
in the information of a new peptide bond and release of the s-RNA that
has been previously attached to the end of the growing peptide chain.
The polypeptide chain is linked to the s-RNA through a new amino acid.
The polyribosome bound aminoacyl-s-RNA and the peptidyl-s-RNA are
therefore intermediates in the protein synthesis.

According to the hypothesis the information for specifying amino
acid sequences, which resides in the DNA polynucleotide is transferred
to protein through m-RNA. The linear sequence of nucleotides in DNA
"cistron" specifies the linear sequence of amino acids in a polypeptide
chain. Messenger-RNA serves as the carrier of information from DNA to
the protein synthesizing apparatus. If the four nitrogenous bases, adenine,
guanine, cytosine, and thymine were to specify the twenty amino acids,
each amino acid would have at least a "3-base code" (175). Code is a
system of arbitrarily chosen words. This would give $4 \times 4 \times 4$ or 64 code
sequences for the amino acids. Evidence has been collected for this
theory by isolating polypeptides consisting exclusively of certain amino
acids from cell-free systems in which synthetic polypeptides are formed
(118). Recently all the possible trinucleotides were tested extensively
for their ability to stimulate the binding of labeled amino-acyl-s-RNA to
ribosome (152), by an elegant new technique (116), and a total of 56 such
code sequences were established. Mechanisms involving
the movement of ribosomes along m-RNA co-ordinated with peptide chain synthesis are postulated (119, 171).

RNA IN THE NUCLEUS

Experimental results from various sources indicate that most of the cellular RNA is synthesized in the nucleus. The specific functional role of nucleoli in normal and neoplastic cells have not been clearly defined. A great mass of evidence has been accumulated which suggests that the nucleolus plays a predominant role in RNA synthesis in the nucleus (19). The role of the nucleus itself as a primary site of RNA synthesis has been established by a number of experimental studies. In one of the most definitive studies, Goldstein and his colleagues (39-41) showed that the nucleus of protozoal cells is the first part which is labeled by precursors of RNA such as purine and pyrimidine nucleosides. During the first hour following exposure of the cell to tritiated uridine or cytidine, the label was found only in the RNA of the nucleus. It was transferred from the nucleus to the cytoplasm over a period of 24 hours, and at that time, the label was distributed between the nucleus and the cytoplasm. Such findings suggest a role for the nucleus as a site of biosynthesis of RNA. Evidence that the pathway was unidirectional was obtained from studies in which labeled nuclei were transferred to unlabeled
amebas. The isotope again moved from the nucleus to the cytoplasm; but was not transferred back to the unlabeled nucleus.

Evidence of the important role of the nucleolus in biosynthesis of RNA has arisen from a number of types of studies.

Autoradiographic studies on labeling of cells gave evidence for rapid early labeling of RNA in the nucleolus, in studies with tritiated cytidine or uridine (19). In these studies, the most concentrated labeling in the cell was found in the nucleolus, although other fractions of both nucleus and cytoplasm had a higher total amount of isotope. From such studies it can be concluded that the nucleolus contains an RNA fraction that turns over at a very rapid rate. The high specific activity of this fraction has been interpreted to mean that nucleolar RNA is a precursor of either nuclear or cytoplasmic RNA or both.

Using the technique of ultraviolet microbeam irradiation, Perry et al. (124, 125) suppressed nuclear function. They carried out studies of incorporation of labeled precursors into cytoplasmic and nuclear RNA, following the "enucleolation" of cells. They found that labeling of nuclear RNA was suppressed about 30% when the nucleolus was irradiated. Labeling of the cytoplasmic RNA was suppressed 70% under the same conditions. These data suggest that RNA synthesized or modified
in the nucleolus accounts for a large proportion of the RNA synthesized in the nucleus and particularly that which is transferred to the cytoplasm.

The great enlargement of the nucleolus which can be caused by thioacetamide has been interpreted to indicate that this compound produces a block in the release of nucleolar RNA (88). It may be presumed that if such an in vivo metabolic block was indeed occurring in the presence of thioacetamide, some knowledge might be obtained of the function of the RNA in the nucleolus. Two major phenomena occur in the cytoplasm of livers of animals treated with thioacetamide. One is a very marked decrease in the protein and RNA content of ribosomes and mitochondria along with a decreased turnover of both protein and RNA in these fractions. A very marked increase in the amount of cytoplasmic proteins and one of the components of cytoplasmic RNA is also observed. These findings support the concept that the nucleolus has an important role in the biosynthesis of cytoplasmic ribosomes or some component of the ribosomes and that inhibition of nucleolar activity results in suppression of this biosynthetic function.

The isolation of RNA molecules from mammalian tissue was relatively unsatisfactory (96) before the introduction of Kirby's phenol extraction method (82). This method consists of extracting the RNA from the tissue fractions by aqueous phenol. The main advantage of this
method is that DNA generally is not solubilized and extracted with the RNA. The RNA prepared by this method has been reported to be virtually protein free (82). This method is eminently suitable for the preparation of RNA from the nuclei.

Several modifications of the phenol extraction procedure have been reported. Detergents have been widely used in purifying RNA from a variety of mammalian, plant and bacterial sources. Detergent deproteinizes the RNA from the ribosomal particles. Among the reagents most commonly employed are the ionic steroid, sodium deoxycholate (168), a non-ionic detergent which is a copolymer of polyoxyethylene and cetyl alcohol (lubrol W), sodium dodecyl sulfate, naphthalene disulfate and 8-hydroxyquinoline (26). The pH of the extracting medium has an influence on the amount (5) and the base composition (16) of the RNA extracted. The RNA extracted by phenol is precipitated from the aqueous layer by the addition of alcohol and sodium chloride.

The relationship between RNA and the large variety of proteins which are synthesized in a single cell suggest the heterogeneity in intracellular RNA. The nucleus is not only the site of RNA synthesis but also the site of synthesis of nucleoproteins. The RNA of the nucleus may then be expected to be heterogeneous. In addition to this inherent
heterogeneity, there was also a superimposed "artifact" heterogeneity which may arise during the isolation of the nuclear RNA. The current interest in nucleic acids has resulted in an intensive search for new methods of fractionation of various nucleic acids and their breakdown products according to the metabolic activity, molecular size, and base composition.

There are many reports that RNA of the nucleus could be resolved into different fractions having varying rates of labeling or base composition. Several techniques including phenol treatment, the use of different kinds of column chromatography and by the use of sucrose density gradient centrifugation (108, 25, 43, 92, 110, 122, 141, 156, 174) have been used.

Sucrose density gradient centrifugation has been shown to be a useful method to fractionate RNA. A preparation of nuclear RNA has been sedimented as two components (56), one having a sedimentation coefficient of approximately 20 S and the other having about 16 S. The molecular weights of these two fractions were calculated to be about $1.3 \times 10^5$ and $6 \times 10^5$ respectively. RNA has been isolated from liver nuclei (156) and fractionated into four fractions (40 S, 33 S, 19 S, and 6 S) by much the same method. The sedimentation coefficient so obtained is a function of both size and shape of the molecule. If the shape factors
are assumed to remain constant, RNA molecules with higher sedimentation coefficients have higher molecular weights (156). When these fractions were assayed, all four of them possessed m-RNA activity to various degrees. Nuclear RNA and nuclear RNA contaminated with significant amounts of cytoplasmic RNA gave an essentially identical sedimentation pattern (156). Sedimentation of rapidly labeled RNA fraction of nuclei showed radioactivity in all fractions in varying amounts (156). The same results were obtained when RNA from various other sources were subjected to sedimentation analysis (79, 111, 114, 159).

Fractionation of RNA has been achieved by chromatography on methylated albumin columns (25, 110, 122). Here again four fractions could be obtained when nuclear RNA was subjected to this technique (107, 110, 136). The higher the salt concentration used for the elution, the higher was the sedimentation coefficient of the RNA fraction eluted from the column. The chromatographic behavior of the RNA appears to depend on molecular weight and probably also on nucleotide composition (110).

Cellulose treated with epichlorohydrin and triethanolamine (Ecteola) has been used as an anion exchanger for fractionating RNA (15, 43, 141). The elution profile differs significantly to RNA mixtures.
from different sources as well as from the same source isolated by different methods. The fractions eluted from Ficoll columns were subjected to sedimentation analysis. It has been found that RNA fractions eluted at higher salt concentrations have higher mean sedimentation coefficients (141-147).

Other column chromatographic procedures for the fractionation of RNA have been described. Several authors have used calcium phosphate columns and demonstrated the separation of RNA into four principal fractions (92, 174). DEAE-cellulose (see Appendix III) columns are used for the fractionation of RNA with similar results (132, 145, 184). Fractionation by counter-current distribution (78) and by temperature or salt concentration gradients applied to columns of DNA-agar with RNA-DNA or DNA-DNA duplexes also have been used (102, 103).

**RNA IN THE WOUND TISSUE**

The nucleic acids undoubtedly are intimately connected with the synthesis of collagenous protein in fibroblasts. Nucleic acids of fibroblasts probably do not have any direct relation to the extracellular fiber formation, but are closely associated with the intracytoplasmic phase of collagen synthesis. RNA in the wound tissue has been less extensively investigated. However, there are a few reports concerning the metabolism of nucleic acids in the granulating wound
Tsenev (164-167) studying the role of RNA in the wound healing process, found that the lesion (injury) of the tissue leads to a rapid disaggregation of the RNA. He suggested that the disaggregation products of RNA have a favorable effect on the wound process expressed in accelerating the biological cleansing of wounds and contributing to the formation of the granulation tissue. This conclusion was confirmed by the clinical testing of a preparation containing RNA derivatives.

Hosoda (69) measured the nucleic acid phosphorus in the regenerating wound tissue. He found that the content of both nucleic acids (DNA and RNA) gradually increased up to the 8th day after wounding, then tend to diminish slowly. Although the nucleic acid content was highest on the 8th day, incorporation of radioactive phosphorus into wound tissue gave two peaks. The first peak appeared on the fourth day and the second peak on the 8th day. RNA always showed a higher incorporation of $^32$P than DNA. The greatest incorporation and the high level of specific activity of RNA, 8-10 days after wounding, correlates with the phase of active fibroblastic proliferation and new fiber formation. The rapidly increasing accumulation of insoluble collagen on the 10-12th days infers preceding formation of precursor collagens. Thus Hosoda concludes that it is not unreasonable to imagine
that the rapid incorporation of $^32P$ into nucleic acids, when the active proliferation of fibroblasts exists, is a reflection of collagen synthesis at its maximal level.

Ribonucleic acid changes during development of polyvinyl sponge implant connective tissue were studied by extracting the RNA by aqueous phenol (10). It was found that there was a continuing decrease of RNA per cell from day 8 to day 20 of tissue development. This change was qualitatively parallel to a decrease in cellular synthesis of collagen.

Williamson and Guschlbauer (179) have studied the metabolism of nucleic acids in the regenerating wound tissue in relation to its deposition, relative distribution in various subcellular fractions and rate of formation. The total cellular RNA was found to be formed most rapidly during the early stages of regeneration; the rate of formation decreasing with time. But the peak concentration of total RNA appeared at about 7-10 days after wounding. The incorporation of $^32P$ into the RNA of the ribosomes followed a similar pattern. However, the RNA of the nuclei of the cells appeared to be formed more rapidly as regeneration progressed. The rapid formation of the ribosomal RNA suggested that it is utilized for cellular protein formation because the period of rapid incorporation coincides with the period of rapid and extensive cell proliferation in the regenerating tissue (58). On the other hand, the RNA in the cellular nuclei appeared to be formed much less rapidly than that
in the ribosomes. The slow rate of formation of this RNA and the deposition of collagen about 7 days after wounding made them suggest that there would be a possible metabolic connection between these two. A change in the purine of pyrimidine ratio of the total cellular RNA of the regenerating wound tissue with time, supported the concept of a change in the predominant types of RNA in the cells of regenerating tissue (177).

Williamson and Hertel (180) isolated the nuclear material from regenerating wound tissue and the nuclear RNA was separated from DNA. The isolated RNA was hydrolyzed, the nitrogenous bases and nucleotides were separated by ion-exchange chromatography and the purine to pyrimidine ratio determined. It was found out that the ratio calculated was somewhat lower than those previously published (69) for the total cellular RNA of regenerating wound tissue.
CHAPTER IV

THE PROBLEM

According to the current theory of protein synthesis and to the results of the investigations of the nuclear RNA, it would seem that at least two groups of RNA molecules are present in the cellular nuclei (94, 135, 146). One group of nuclear RNA is concerned with nuclear protein synthesis consisting of nuclear m-RNA, r-RNA and s-RNA. The other group consists of various m-RNA molecules necessary for the cytoplasmic protein synthesis. The nuclei of fibroblast cells of regenerating wound tissue are expected to contain, in addition to the above mentioned RNA molecules, the m-RNA molecules concerned with the biosynthesis of collagen precursors.

Earlier investigations have shown that there is a close relationship between the total cellular RNA of wound tissue and active collagen synthesis (69, 179). Available data on the RNA in the experimental granulation tissue show that the maximum values are reached at 7-10 days after wounding (69, 179). It was also demonstrated with P incorporation studies that RNA of the nuclei of the wound tissue cells is formed rapidly at the time of active collagen formation (179).

Determination of the base composition of total cellular RNA from regenerating wound tissue showed a decrease in purine / pyrimidine ratio
Analysis of the nuclear RNA from this tissue showed even further decrease in purine/pyrimidine ratio (80).

The nature of the relation between collagen formation and the RNA molecules involved is, however, unclear. The purpose of the present investigation is to obtain further information about the nuclear RNA during the formation of granulation tissue. Specifically the relationship between RNA and the collagen synthesis will be investigated.

For this purpose, answers to the following questions will be sought:

I. Can the nuclear RNA of wound tissue be isolated and fractionated by previously reported techniques?

II. What are the characteristics of the nuclear RNA of regenerating wound tissue at different stages of formation with regard to:
   a) Base composition?
   b) Rate of formation?
   c) Molecular size?

III. Can some characteristics of nuclear RNA be related to collagen?
CHAPTER V

EXPERIMENTAL PROCEDURES AND METHODS

A. EXPERIMENTAL PROCEDURES:

Mature female rats of Sprague Dawley Strain were used in all experiments. They weighed generally about 190 - 20 gms at the beginning of the experiment. The rats were housed in individual cages and fed a protein-free diet (see Appendix I). Water was allowed ad libitum. They were weighed at regular intervals during the course of the experiments. Within the experimental period (5-12 days) the animals lost from 5-10 gms in weight. To study the change in nucleic acid composition in the regenerating wound tissue, experiments should be conducted at different stages of regeneration. In order to have a long enough interval between the experimental healing stages (5th, 8th, and 12th days after wounding), a slow process of healing tendency would be required. To insure slow healing, diets completely free of protein were given to the animals.

Wounding Procedure: After an acclimatization period of 2-3 days in the individual cages on the protein-free diet, the animals were anesthetized by keeping them in an ether chamber. When they were completely anesthetized, the outline of a coin (4 cm. in diameter) was traced in the scapular region and the skin was excised down to the muscle along
the outline (50, 63). The skin wound was blotted with cotton, wet with
70% ethanol. The animals were put back in individual cages and fed
the same diet. Wounds were allowed to heal without any further
attention.

**Harvesting of the Wound Tissue:** The animals were anesthetized as
before and the fibrin clot on the regenerating wound tissue was removed
with forceps. After the wound was cleaned superficially with absorbant
cotton, wet with saline, the regenerated tissue of the wound was cut
out. Care was taken not to include skin or underlying fascia and muscle.
The tissue was immediately weighed and frozen in dry ice-dupanol.
It was stored in air-tight polyethylene bags at -18°C.

**Isolation of the Nuclear Material:** The isolation of intact nuclei was the
first step in an investigation of nuclear RNA. Frequently it was found
that the separation of nuclei from tissue under examination is by no
means easy. A useful review of methods for the particular problems in
the isolation of nuclei from different types of cells have been discussed
by Dounce (32).

The basic of many procedures for the isolation of nuclei depends
upon the homogenization of the tissue followed by differential centri-
fugation. Sucrose solutions are usually used for the homogenization
of the tissue. The importance of the concentration of metal ion (usually calcium or magnesium) in the homogenizing medium has been clearly illustrated by electron microscopy in the careful work on the isolation of nuclei from guinea-pig liver by Maggio et al. (97). Chauvean et al. (23) centrifuged the tissue homogenate on a density gradient to isolate nuclei. In general, the density gradient centrifugation of an appropriate tissue homogenate is considered to be the most satisfactory means of purification of cell nuclei.

The isolation of the nuclear material from regenerating wound tissue becomes difficult because of the presence of large amounts of collagen fibers. Different methods have been tested for homogenizing the tissue.

The wound tissue was frozen in liquid nitrogen and crushed by repeated blows of a hammer on steel pestle in a cold steel mortar. The crushed tissue was then transferred to a chilled flask and suspended in a sucrose medium and centrifuged. The nuclei isolated by this procedure were found to be broken and embedded in cytoplasmic material when histologically examined. Moreover the procedure was tedious and undependable.

Homogenization of the wound tissue in a glass tissue grinder was found to be most satisfactory for the isolation of nuclear material.
The nuclei thus isolated were found to be largely intact and mostly free from cytoplasmic material when histologically examined.

According to this procedure the frozen wound tissue was suspended in two volumes of 0.25 M sucrose solution containing 0.003 M CaCl\textsubscript{2} prepared in 0.05 M Tris (see Appendix III) buffer at pH 7.2. It was homogenized at 4°C for 15 minutes with the aid of a drill press. The homogenate was suspended in 20-25 volumes of the same medium and shaken in the cold for 1 hour and the suspension was centrifuged for 20 minutes at 2500 rpm. in the cold. The supernatant was discarded and the residue which contained the nuclei along with a few unbroken cells and collagen fibers, was resuspended in two volumes of the same medium and homogenized for 5 more minutes. It was again suspended in approximately 25 volumes of sucrose solution and shaken in the cold for 45 minutes. The homogenate was centrifuged and the residue was washed with 10 volumes of sucrose solution. A portion of this preparation was stained in Eosin-Hematoxylin and examined under a microscope. Pictures taken of these slides are given in Fig. I.

Examination of the stained nuclear preparation revealed that the nuclear residue consisted of intact nuclei contaminated with large amounts of collagen fragments and slight amounts of cytoplasmic debris.
Fig. 1: The picture was taken from a stained smear of a nuclear preparation of wound tissue. The wound tissue obtained from rats, 8 days after wounding, was homogenized in sucrose solution and the nuclear material was isolated with collagen by differential centrifugation. A smear, prepared from the nuclear material, was stained first in hematoxylin for nuclei and then in eosin for cytoplasmic material. The arrows point to the whole and fragmented nuclei and the long dark lines and areas seen in the picture represent collagen fibers and broken pieces of collagen (magnified 915 times).
Further attempts to purify this preparation met with little or no success. However, the use of these crude fractions without further purification for the isolation of nuclear RNA could be justified. Collagen particles would not interfere with the isolation of nuclear RNA because the procedure (phenol extraction) is not likely to solubilize any protein or collagen. A nuclear preparation from which nuclear RNA is to be isolated should be absolutely free of cytoplasmic contamination.
Even after repeated homogenization, washing and centrifugation, the nuclear residue was not completely free of cytoplasmic contamination. Under conditions of the preparation described, the cytoplasmic contamination of the nuclear fraction appeared as shreds of material. But no discrete particles such as mitochondria or microsomes could be detected. The homogenization technique employed was not expected to result in extensive destruction of intact mitochondria or microsomes. Consequently no significant contamination by cytoplasmic RNA was to be expected. Therefore, no appreciable contamination by microsomal RNA and soluble RNA would be expected in the nuclear RNA isolated from the nuclear material.

**Extraction of the Crude Nuclear RNA:** In order to study the characteristics of nuclear RNA, a pure and intact nuclear RNA preparation is required. The common method adopted for isolating RNA from cells or subcellular components is phenol extraction (82). It consists essentially of shaking the nuclei with aqueous phenol. The RNA is released into the aqueous medium. It is separated by centrifugation and the RNA precipitated with 60-70% ethanol and 1% sodium chloride. The release of RNA by phenol is not quantitative. Some RNA is left in the interphase emulsion which forms between aqueous and phenol phase during centrifugation. This RNA, usually called "interphase RNA", can be released into aqueous phase by
another phenol extraction of the interphase material at 60 °C. (149, 183).

RNA extracted at 60 usually becomes degraded. Therefore, all extractions of the nuclear RNA were carried out at 4 °C. To release the "interphase RNA" at this low temperature, the nuclear material was extracted repeatedly with more and more alkaline solutions.

The nuclear material was made to 10 ml. volume in 0.05 M Tris buffer at pH 7.2 in a volumetric flask. Aliquots were removed for tyrosine determination. To the remaining nuclear suspension there was added 1 ml. of 1% lauryl sulfate solution to rupture the nuclear membrane. It was shaken for 30 minutes at 4 °C. The shaking was continued for 60 more minutes after adding 10 ml. of 88% liquid phenol and the whole mixture was centrifuged at 25,000 x g for 45 minutes in the cold. The supernatant aqueous phase was collected and the phenol and interphase emulsion was extracted in the same way twice more with 5 ml. portions of buffer at pH 7.2. The phenol and the interphase emulsion were similarly extracted three times with 0.05 M Tris buffer at pH 8.6 and finally three times with 0.05 M KOH.

The absorbance of the extracts was measured at 260 mA and the RNA was precipitated in 60-70% ethanol and 1% NaCl. The O. D. units and the ease of precipitation of the extracts obtained from a trial experiment are given in Table II.
**TABLE II**

**RECOVERY OF RNA IN EXTRACTS OF NUCLEAR MATERIAL**

<table>
<thead>
<tr>
<th>Extracting Medium</th>
<th>Extraction No.</th>
<th>O. D. Units (260 mÅ)</th>
<th>Precipitability by 70% alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral solution (pH = 7.2)</td>
<td>1</td>
<td>5.28</td>
<td>precipitable</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.68</td>
<td>precipitable</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.89</td>
<td>not precipitable</td>
</tr>
<tr>
<td>Weakly alkaline solution (pH = 8.6)</td>
<td>1</td>
<td>4.55</td>
<td>precipitable</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.52</td>
<td>precipitable</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.89</td>
<td>not precipitable</td>
</tr>
<tr>
<td>Strongly alkaline (0.05 M KOH)</td>
<td>1</td>
<td>Not measured</td>
<td>precipitable</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>not precipitable</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>not precipitable</td>
</tr>
</tbody>
</table>

* Wound tissue from 15 animals, 5 days after wounding, was used for this pilot experiment.
Thus, the nuclear material was extracted from phenol three times with 0.05 M Tris buffer at pH 7.2 with Tris buffer at pH 8.6 and with 0.05 M KOH. The three extracts made with each solvent were pooled and the RNA was precipitated in 60-70% ethanol and 1% NaCl. It was kept at 0°C for at least 24 hours in order to permit complete precipitation.

A preparation of nuclear RNA of liver nuclei by this method was reported to have some contamination of DNA (141). The DNA was removed by treating the preparation of nuclear RNA with deoxyribonuclease (DNase). The crude preparation was dissolved in 5 ml. of 0.05 M Tris buffer at pH 7.2. It was treated with 50/mg of DNase (crystalline, Worthington Biochemical Corporation) and 0.2 ml. of 0.002 M MgCl₂ for 20 minutes at room temperature (141). The RNA was reprecipitated with ethanol. It was stored under alcohol at -10°C until it was fractionated.

To obtain enough RNA for the various analytical procedures to be described, it was necessary to pool wound tissue from at least 20 animals. The isolation of nuclear material from the wound tissue samples and the extractions of the RNA were carried out as one continuous operation and required approximately 35-40 hours for completion.

Fractionation of Nuclear RNA: The heterogeneity of nuclear RNA is evidently due to the presence of nuclear m-RNA, r-RNA and s-RNA.
The different RNA moieties may have different molecular weights, composition and function. Among the available methods of fractionation are sucrose density centrifugation (56), chromatography on methylated albumin columns (99), Ecteola columns (141), calcium phosphate columns and DEAE cellulose columns (134). Using these techniques, it was possible to fractionate liver and thymus RNA into 3-4 fractions.

Attempts were made to apply some of these methods to fractionate nuclear RNA of regenerating wound tissue. Methylated serum albumin columns were prepared essentially according to Mandel et al. (99). A sample of nuclear RNA was applied to the column. It was eluted with a LiCl gradient (500 ml. of 1 M LiCl mixed with 250 ml. 0.1 M LiCl). Two distinctly different fractions could be eluted. It was followed by another fraction when the column was finally eluted with 2 M LiCl. It was clear that complete elution of the nuclear RNA was not possible with 1 M LiCl from the methylated albumin column. Another sample, eluted with a higher salt gradient (500 ml. of 4.0 M LiCl mixed with 250 ml. of 0.1 M LiCl), also came out as two fractions. The absence of a third peak with further elution of the column with higher concentrations of LiCl indicated that it might have been eluted along with the first two. No further modifications of the procedure were tried due to the fact that it did not separate the nuclear RNA into more fractions and the elution was quite slow.
Attempts were also made to fractionate nuclear RNA on DEAE cellulose, Sephadex, Bio-gel and on mixed columns of Ecteola-DEAE and Sephadex-DEAE. The results were not any better than those obtained on the methylated serum albumin column.

The best results appeared to be achieved by fractionation on Ecteola (see Appendix III) columns. Ecteola was prepared by suspending two gms of the anion exchanger (fine mesh, 0.4 mg./Gm. capacity) in 100 ml. of 1 M LiCl prepared in 0.05 M KOH by shaking. After settling, the clear liquid was decanted off. The washing procedure was repeated two more times with the same solutions. It was then washed three times with 100 ml. of 0.05 M Tris buffer at pH 7.2. The washed resin was suspended in 100 ml. of the above buffer and poured into a funnel which was attached to a 30 x 1 cm. column filled with Tris buffer. It was then allowed to settle by gravity. The narrow outlet of the column was connected to a Vanguard ultraviolet analyzer by gumrubber tubing and the analyzer was set to read absorbance at 260 m\(\mu\). After packing, the top of the column was connected to a reservoir containing 0.05 M Tris buffer at pH 7.2 with a head pressure of about 1.25 meter. The column was washed by passing the buffer through the column until the effluent ceased to absorb U. V. light at 260 m\(\mu\). As measured the final dimensions of the packed column were approximately 18 x 1 cm. and it had a flow rate of about 30 ml. per hour.
The crude nuclear RNA was dissolved in 2.0 ml. of 0.05 M Tris buffer at pH 7.2. An aliquot of 0.2 ml. was removed to measure the total absorbance and the remainder was applied on the column.

Elution of the RNA with a continuous concentration gradient fractionated it into two peaks. This was more or less the same as those obtained by Reiner et al. (141). However when a discontinuous salt gradient was used for the elution, four distinct fractions could be obtained with 0.2 M, 0.4 M, and 0.6 M LiCl in Tris buffer and 1.0 M in 0.05 M KOH.

The column was washed with neutral Tris buffer and another sample of nuclear RNA was applied. Stepwise increasing concentrations of 0.2 M, 0.4 M, 0.6 M, 0.8 M, 1.0 M and 4.0 M LiCl in 0.05 M Tris buffer, pH 7.2 and 1.0 M, 2.0 M and 4.0 M LiCl in 0.05 M KOH were passed through the column. The absorbance was automatically recorded.

Four ml. fractions were collected and the absorbance of these fractions was also measured on a Beckman DU Spectrophotometer at 260 m. The fractionation was carried out at room temperature. After regenerating the column with buffer it was used over and over again.

Recovery of the RNA Fractions: The RNA fractions had to be recovered from the LiCl solutions for further analysis. The sample tubes of each RNA fraction were combined and the volume was reduced to 2-3 ml. by freeze drying. All the nuclear RNA fractions were precipitated with
alcohol and kept at -10°C until further analysis was carried out.

Figure II shows the fractionation pattern of crude nuclear RNA on Ecteola column. The RNA was obtained from the wound tissue on the 5th day after wounding. It was extracted from nuclear material with 0.05 M Tris buffer at pH 7.2. As seen in the diagram no RNA appeared in the wash eluant of the buffer. Fractions I, II, and III were eluted with 0.2 M, 0.4 M and 0.6 M LiCl respectively in 0.05 M Tris buffer. The elution was continued by 0.8 M, 1.0 M, 2.0 M, and 4.0 M LiCl in the same buffer (elutions with 2.0 M and 4.0 M LiCl were not shown in the diagram). No more RNA appeared at this pH and with LiCl concentrations higher than 0.6 M. When the column was eluted with 1.0 M LiCl in 0.05 M KOH, Fraction IV was obtained. Further elution with 2.0 M and 4.0 M LiCl (not shown in the diagram) in 0.05 M KOH did not yield any RNA fractions. All the RNA fractions started eluting with the first 5 ml. of the respective eluates and completed with in 20-40 ml. Thereafter, up to 200 ml. of the same eluting solution yielded no further RNA. Only four fractions could be eluted from each crude nuclear RNA preparation fractionated.

Estimation of Approximate Size of the Nuclear RNA Fractions: The fractionation on Ecteola column has shown that the nuclear RNA of regenerating wound tissue is heterogeneous. The fractionation results indicate the existence of different groups of RNA molecules of widely
Fractionation of crude nuclear RNA isolated from wound tissue on the 5th day after wounding and extracted from phenol suspension of nuclear material with neutral Tris buffer (pH = 7.2). The RNA was placed on the Ecteola column and eluted with different concentrations of LiCl as described in the text.
different molecular size. In the absence of an analytical centrifuge, precise information of the size of the RNA components could not be obtained. However, an attempt was made to find out the approximate molecular size of the nuclear RNA fractions by the use of Bio-gel columns (6).

Five hundred milligrams of Bio-gel were weighed and suspended in distilled water by shaking. It was kept over night at room temperature. The gel was poured into a 30 x 1 cm. column and allowed to settle by gravity and the finished column was adjusted to 15 cm. long. After packing, the column was washed with large volumes (100-150 ml.) of distilled water until the effluent ceased to absorb U. V. light at 260 mÅ. The outlet of the column was connected to a fraction collector which was equipped to collect 5 ml. fractions.

The RNA fraction obtained from Ecteola was dissolved in 0.05 M Tris buffer at pH 7.2 and made up to a volume. An aliquot was applied to the column. It was eluted with water and 5 ml. fractions were collected. The absorbance of each fraction was measured at 260 mÅ. Molecule size calculations are made with the percentage recovery of the RNA fractions from the column. The column was washed with distilled water and used over and over.
The above procedure was carried out for all nuclear RNA fractions obtained from Ecteola column and the RNA fractions were passed through different kinds of Bio-gel at room temperature. The different kinds of Bio-gel are represented by the notation "Bio-gel - P - X".

where "X" stands for the size of the gel bead which would absorb molecules of size "X" x 10^3 or less. For example, Bio-gel - P - 30 represents gel beads which would absorb molecules of size 30 x 10^3 or less. Molecules of higher than 30,000 molecular weight will pass through the column without any delay. The molecules which are absorbed to the column can be eluted by passing excess amounts of water or dilute buffer through the column.

The Rate of Formation of Nuclear RNA Fractions: Nuclei are the main site of the bulk of RNA synthesis (55, 133, 151). They incorporate labeled precursors of RNA more rapidly than the cytoplasm (9, 55, 133, 151). In previous studies (179), it has been shown that the nuclear RNA has the highest rate of formation in regenerating wound tissue. Fractionation of nuclear RNA of wound tissue on Ecteola column shows that it is composed of many RNA components. The objective of this experiment is to find out the relative rate of formation of these RNA fractions.

Nuclear RNA from regenerating wound tissue, on the 5th, 8th, and 12th day of regeneration, showed more or less the same fractionation pattern. The RNA content of wound tissue increases with time and reaches a
maximal concentration in approximately 7-10 days after regeneration begins (69, 177). Since the incorporation and turnover of $^3P$ in nuclear RNA parallels the deposition of collagen in wound tissue (179), the rate of formation of each RNA fraction on the 8th day of regeneration was studied.

Studies with labeled precursors of RNA have shown that the concentration of the label in the nucleus reaches a maximum within one hour following the exposure of the cell to the label (39-41). Since the label gets out into the cytoplasm after one hour, nuclear RNA of regenerating wound tissue was extracted and fractionated after exposing the animals 10, 20, 35 and 55 minutes to tritiated uridine.

Eighty four animals were wounded and kept on the protein-free diet. On the 8th day after wounding, the animals were anesthetized by subcutaneous injections of nembutal. Each animal received 115/4 curie of tritiated uridine (supplied by Schwarz Bio-Research, Inc., Sp. Act. = 4 c/mM) by subcutaneous injections. The wound tissues were collected from each batch at intervals of 10, 20, 35, and 55 minutes after the administration of the labeled uridine. From the wound tissue the nuclear RNA was isolated and fractionated as described before. The radioactivity of each fraction was counted in a Packard Tri-Carb Liquid Scintillation Spectrometer according to the procedure described in the section on analytical procedure.
B. ANALYTICAL PROCEDURE

Tyrosine Determination: A modified Folin-Ciocalteu method (34) was used. Three milliliters of 0.1 M NaOH were added to 0.2 ml. of nuclear material from the wound tissue homogenate in a glass stoppered centrifuge tube. It was heated on a boiling water bath for 20 minutes and cooled to room temperature. The solution was made to 5.0 ml. It was cloudy. Centrifugation did not reduce the cloudiness. Aliquots of 1.0 ml. of this slightly cloudy solution were used for the tyrosine determination.

To 1.0 ml. aliquots of standard solution and samples in glass stoppered test tubes were added 1.0 ml. portions of carbonate-copper solution (see Appendix III). The tubes were shaken well and allowed to stand for 10 minutes at room temperature. To this mixture there was added 0.1 ml. of Folin-Ciocalteu reagent (see Appendix II). After mixing, the reaction mixture was made up to 5.0 ml. After 40 minutes the absorption was read against the corresponding blank with a No. 54 filter in a Klett-Summerson Colorimeter. The tyrosine content of the samples was calculated from the standard curve (see Figure III).

Determination of Base Composition: Paper chromatographic procedures were employed to determine the base composition of the various fractions isolated (182). The RNA fractions were hydrolyzed in 6 M HCl by heating in a boiling water bath for 40 minutes in a glass stoppered tube.
Fig. 3: Standard curve for Folin-Ciocalteu reaction. The reaction was carried out as described on page 62. Samples were read at 540 m\(^\lambda\) and the Klett reading plotted against concentration (\(^\mu\)g. /5 ml.) of tyrosine.
The acid was evaporated off under reduced pressure; the hydrolysate was dissolved in 50 lambda of 0.2 M HCl and 40 lambda of it was spotted on 20 x 1 inch Whatman No. 1 filter paper strips. The chromatogram was developed in isopropanol-HCl-water system, prepared by adding enough water to a mixture of 65 ml. redistilled isopropanol and 16.7 ml. conc. HCl to make 100 ml. (182) for 24 hours. After developing the chromatogram was dried at room temperature and the bases were detected by a source of U.V. light at 260 m\(\mu\). The detected bases and nucleosides were cut out and suspended in 3.0 ml. of 0.2 M HCl in glass stoppered tube. It was shaken for 90 minutes. After centrifugation, to sediment the paper shreds, the absorbance of the clear solution was measured at 250 m\(\mu\) for guanine, 260 m\(\mu\) for adenine and uridine, and 280 m\(\mu\) for cytidine. For blank, 8-10 ml. of 0.4 M LiCl in 0.05 M Tris buffer at pH 7.2 was freeze-dried and treated the same way as the samples. Adenylic acid, guanylic acid, cytidylic acid and uradylic acid were hydrolyzed as above. Different amounts were chromatographed separately for the standard curves. Figures IV, V, VI, and VII show the standard curves for adenine, guanine, uracil and cytidine, respectively. The amounts of the purine bases and pyrimidine nucleosides are determined from the standard curves.
Fig. 4: Standard curve for adenine. Samples were chromatographed as described on page 64. It was read at 260 m\(\mu\) and the optical density plotted against concentration (\(\mu\)moles/3 ml.) of adenine.
Fig. 5: Standard curve for guanine. Samples were chromatographed as described on page 64. It was read at 250 m\(\mu\) and the optical density plotted against concentration (\(\mu\) moles/3 ml.) of guanine.
Fig. 6: Standard curve for uridine. Samples were chromatographed as described on page 64. It was read at 260 mÅ and the optical density plotted against concentration (μmoles/3 ml.) of uridine.
Fig. 7: Standard curve for cytosine. Samples were chromatographed as described on page 64. It was read at 280 m\(\lambda\) and the optical density plotted against concentration (\(\mu\) moles/3 ml.) of cytosine.
A recovery experiment was conducted as follows. Known quantities of each of the four nucleotides were mixed. The mixture was chromatographed according to the above procedures. The quantity of each base was obtained from the standard curves. The recovery was calculated. The results are shown in Table III.

**Determination of Radioactivity:** The original conception was to count the \(^3\)H-uridine after the paper chromatography. But when \(^3\)H-uridine, mixed with other cold nucleosides and nucleotides, was subjected to hydrolysis procedure, the radioactivity disappeared. It was found experimentally that when the \(^3\)H-uridine was heated in acid solution, the tritium exchanged with the hydrogen ion of the medium. Therefore, the radioactivity had to be measured before the RNA fractions were hydrolyzed in HCl. In order to measure the uridine to calculate the specific activity, the RNA fractions were recovered from the scintillation fluid. This was done by reprecipitation with 95% ethanol from the Hyamine medium. The following procedure was adopted.

The RNA fractions obtained by elution from the Lcteola columns were dissolved in 1.0 ml. of 0.1 M Hyamine in absolute methanol. The mixture was transferred to counting vials and 10 ml. of the scintillation fluid (see Appendix II) added. It was counted for 10 minutes in a Tri-Carb Liquid Scintillation Spectrometer. For standard, a sample of the tritiated uridine was used. The samples were always kept at 4 °C.
**TABLE III**

**RECOVERY OF NUCLEOTIDES FROM PAPER CHROMATOGRAPHY**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>μ moles added</th>
<th>μ moles recovered</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenyllic acid</td>
<td>0.049</td>
<td>0.050</td>
<td>102</td>
</tr>
<tr>
<td>Guanylic acid</td>
<td>0.052</td>
<td>0.052</td>
<td>100</td>
</tr>
<tr>
<td>Cytidylic acid</td>
<td>0.053</td>
<td>0.056</td>
<td>105</td>
</tr>
<tr>
<td>Uridylic acid</td>
<td>0.049</td>
<td>0.047</td>
<td>96</td>
</tr>
</tbody>
</table>
After measuring the tritium content of the samples, 30 ml. of acidified 95% ethanol (0.01 M with respect to HCl) was added to precipitate the RNA fractions. It was centrifuged and washed with 5 ml. of cold acid-alcohol. The RNA was dissolved in 1 ml. of 6 M HCl, heated in a boiling water bath for 40 minutes, the hydrolysate was evaporated to dryness under reduced pressure and chromatographed as described in the above section. The uridine content of each fraction was measured and the specific activity of the fraction was calculated.
CHAPTER VI

BASE COMPOSITION OF NUCLEAR RNA OF WOUND TISSUE

AT DIFFERENT STAGES OF REGENERATION.

The objective of this experiment was to compare the difference in amount and composition of nuclear RNA which could be isolated from wound tissue at different stages of regeneration. For this purpose, wound tissue was collected from rats, 5, 8 and 12 days after wounding. The nuclear material from wound tissue was isolated and its tyrosine content measured. The RNA from the isolated nuclear material was extracted with neutral, weakly alkaline and strongly alkaline solutions. These crude extracts of nuclear RNA were fractionated on Ecteola resin. The base composition of the resulting nuclear RNA fractions was determined.

Interpretation of the data obtained from these experiments can best be made after discussing some of the limitations of the techniques employed in isolating the nuclear RNA fractions from wound tissue at different stages of regeneration.

Isolation of the Nuclear Material of Wound Tissue

The isolation of a pure preparation of nuclei would be the prime consideration in studying nuclear RNA. The techniques employed for the isolation of nuclei or nuclear material in general depend on the difference
in specific gravity between the nuclei and the cytoplasmic components of the cell. Since there is a large difference in specific gravity between these cellular components, the isolation of nuclei from the cells in most tissues is rather easy and good yields can be obtained. In granulation tissue, the specific gravity of nucleus is thought to be closer to that of cytoplasm. The nucleus comprises a small fraction of the cell. These properties of the nuclei of the granulation tissue make its isolation difficult.

Since the characteristics of cells in regenerating wound tissue change with time, this poses additional problems for the isolation of nuclear material. Wound tissue after five days of wounding appears to have a high water content. It contains very little collagen and relatively few cell content. The wound tissue, 8 days after wounding, is much firmer. It appears to have a smaller water content, much more collagen and more cells. By the 12th day after the wounding, the wound has contracted appreciably. It has lost even more water. The collagen content has increased even further. But the number of cells has decreased from the previous stage.

Homogenization of the wound tissue to liberate intact nuclei presented further difficulties. The technique (63) of freezing the tissue and crushing it in a mortar with a pestle was tried. It yielded a considerable
degree of fragmentation of the nuclei. The use of a glass tissue grinder was found to be adequate for the homogenization of the wound tissue in ++

0.25 M sucrose solution containing Ca ions. The technique permitted the fragmentation of the collagen fibers. It yielded largely intact nuclei.

Isolation of the nuclei from other cellular constituents of granulation tissue was carried out by centrifugation. It was found difficult to separate the nuclei from collagen fragments. Even after shaking the homogenate in large volumes of sucrose solution, the nuclei were found to be embedded in collagen. However, this procedure helped to release the cytoplasmic material adhering to the intact nuclei. Repeated shaking and centrifugation of the homogenate left the nuclear material almost free of cytoplasmic material. For the isolation of nuclear RNA, nuclear material free of cytoplasm had to be obtained. The presence of collagen in the nuclear material may not matter much since the RNA can be extracted from it. Therefore, it was decided to isolate the nuclei with collagen from wound tissue (nuclear material).

The purity of the nuclear material was estimated by histological examinations. A smear of nuclear material was stained with hematoxylin and eosin (see Fig. 1). It was found to be essentially free of cytoplasmic material and to consist of at least 95% collagen fibers. The remainder seemed to be intact and fragmented nuclei. No whole cells could be found.
The small amount of cytoplasmic contamination could not be completely removed without a considerable decrease in the yield of nuclei.

**Extraction of the Crude Nuclear RNA**

The RNA from the nuclear material on the wound tissue was isolated by a modified phenol extraction procedure (82). In order to have the least possible degradation of the RNA to be isolated, it was thought best to carry out the extractions of the nuclear material at 4 °C, with neutral buffer. Under these conditions, the yield of the nuclear RNA was found to be poor. It has been reported that RNA in the nucleus is complexed with basic proteins (16a). To extract the nuclear RNA in quantitative yield, alkaline buffer solutions are needed. The shaking of the nuclear material with phenol would denature the nucleoprotein complex and make it water soluble. The elevation of temperature and/or alkalinity of the extracting medium would dissociate the denatured complex and liberate the RNA into the aqueous phase (16a). The nuclear material of the wound tissue was extracted with neutral (pH = 7.2), weakly alkaline (pH = 8.6) and strongly alkaline (0.05 M KOH) solutions.

The yield of the crude nuclear RNA from the nuclear extracts of regenerating wound tissue, measured in terms of O.D. Units per mg. tyrosine, is presented in Table IV. The values are only an approximation since the measurements of the absorbance were made at 260 mλ, a
wave length at which not all the purine and pyrimidine of the RNA give
maximal absorption. The values show the total amount of RNA which
could be extracted from the nuclear material at different stages of
formation. Although the values may not be the actual amounts of nuclear
RNA present in the nucleus on a quantitative basis, it is an indication
of the relative amounts of RNA which could be extracted, under the
same conditions, from the nuclear concentrates. The total amount of
RNA reaches the highest concentration 8 days after wounding. Since most
of the RNA is synthesized in the nucleus (37), the level of RNA in the
whole cell may be considered to be a reflection of what is found in the
nucleus. Therefore, the results presented in Table IV are consonant
with the reports that the total RNA content of regenerating wound tissue
reaches a maximum about 7-10 days after wounding (50, 69). This is
the period when there is active fibroblastic proliferation and collagen
formation in the wound tissue. It would seem that there is a definite
relation between the progress of wound tissue formation and RNA
synthesis. Since RNA is synthesized in the nucleus, the relative amount
of nuclear RNA may be expected to be the highest level at this stage
of regeneration. This is found experimentally (Table IV).
### TABLE IV.

**YIELD OF CRUDE NUCLEAR RNA EXTRACTED FROM NUCLEAR MATERIAL OF WOUND TISSUE AT DIFFERENT STAGES OF REGENERATION.**

<table>
<thead>
<tr>
<th>Days after wounding</th>
<th>Amount of RNA obtained by extracting the nuclear material with Neutral solution</th>
<th>Weakly alkaline solution</th>
<th>Strongly alkaline solution</th>
<th>Total yield of nuclear RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>8.4</td>
<td>2.5</td>
<td>2.9</td>
<td>13.8</td>
</tr>
<tr>
<td>8</td>
<td>15.7</td>
<td>3.9</td>
<td>3.8</td>
<td>23.4</td>
</tr>
<tr>
<td>12</td>
<td>5.9</td>
<td>3.0</td>
<td>2.1</td>
<td>11.0</td>
</tr>
</tbody>
</table>

*In terms of O. D. Units per mg. tyrosine in the nuclear protein.*
The relative distribution of the total nuclear RNA into different extracts is found to be about the same at all the stages of regeneration studied. About 55-65% of the total nuclear RNA was found to be extracted with neutral buffer solution. It has been reported that about 70% of the total nuclear RNA may be extracted from liver nuclei with neutral buffer (141). About 20% of the total nuclear RNA was found in each of the crude alkaline extracts of the nuclear material of wound tissue (Table IV).

**Fractionation of Crude Nuclear RNA**

The crude nuclear RNA extracted from nuclear material of wound tissue with neutral, weakly alkaline and strongly alkaline solutions was fractionated on Ecteola. After washing the column with 0.05 M Tris buffer the crude nuclear RNA extract was applied to the column. The washing of the column was continued with another 100 ml. of buffer after applying the RNA sample. No material absorbing at 260 m\(\lambda\) could be eluted from the column with the buffer. RNA fractions I, II, and III were eluted from the Ecteola with 0.2 M, 0.4 M and 0.6 M LiCl in neutral Tris buffer respectively. Washing the Ecteola column with solutions of higher LiCl concentrations prepared in the same buffer did not yield any more RNA. The nuclear RNA, identified as Fraction IV, was eluted with 1.8 M LiCl in 0.05 M KOH. Each RNA fraction began
to appear with the first 5 ml. of eluate; it was completely eluted with the first 50 ml. of eluate. Application of up to 200 ml. of the same solution gave no further material absorbing at 260 m\(\mu\).

The "fractionation profiles" of all the crude nuclear RNA extracts of wound tissue, at different stages of regeneration, on Ecteola column are summarized in Fig. 8. The manner in which the various fractions are to be identified is illustrated in Table V.
Fig. 8: The RNA was extracted from regenerating wound tissue at different intervals (5, 8 and 12 days after wounding) with neutral (pH = 7.2), weakly alkaline (pH = 7.2) and strongly alkaline (0.05 M KOH) solutions. The column was packed, loaded with the RNA and eluted with solutions of LiCl as described in the method section. Descriptions of the RNA fractions are given on the next page.
TABLE V

DESCRIPTION OF THE RNA FRACTIONS

<table>
<thead>
<tr>
<th>Days after wounding</th>
<th>5 = 5 days after wounding</th>
<th>8 = 8 days after wounding</th>
<th>12 = 12 days after wounding</th>
</tr>
</thead>
</table>

Extractions from Phenolic Suspension

Neutral Extracting Solution = 0.05 M Tris buffer, pH = 7.2

Weakly Alkaline Extracting Solution = 0.05 M Tris buffer, pH = 8.6

Strongly Alkaline Extracting Solution = 0.05 M KOH

Fraction I = Eluted with 0.2 M LiCl in 0.05 M Tris buffer

Fraction II = Eluted with 0.4 M LiCl in 0.05 M Tris buffer

Fraction III = Eluted with 0.6 M LiCl in 0.05 M Tris buffer

Fraction IV = Eluted with 1.0 M LiCl in 0.05 M KOH

Illustration: The fraction called "8-8.6-III" is identified in the following way:

- Days after wounding when the wound tissue was harvested
- The pH of the buffer used to extract the RNA from the phenolic suspension of crude nuclear material
- The RNA eluted from the Ecteola column with 0.6 M LiCl in 0.05 M Tris buffer, pH 7.2
- The medium of the buffer used to extract the RNA from the phenolic suspension of crude nuclear material
- The RNA eluted from the Ecteola column with 1.0 M LiCl in 0.05 M KOH
The "elution pattern" of nuclear RNA of wound tissue may be compared with that obtained by Bradly and Rich (15). They have separated RNA, from calf liver and yeast, with different mean sedimentation coefficients on Ecteola. They have found that the higher NaCl concentrations of the eluant, the higher the sedimentation coefficient of the RNA eluted. Even when they used very concentrated salt solutions, a portion of the RNA could not be eluted from the column. This portion could be eluted only with 1.0 M NaOH at room temperature or with 1.0 M NaCl at 95 °C.

This has been interpreted to mean that the residual RNA on the Ecteola column can be removed only by degradation with alkali or by high temperature (15).

The recovery of the crude nuclear RNA extracts of the nuclear material of wound tissue is shown in Table VI. It can be seen that the average recovery of nuclear RNA is about 60%. These results are in agreement with the report that about 60% recovery of brain nuclear RNA was obtained from methylated serum albumin by stepwise elution with increasing concentrations of NaCl (62).

In Table VII is shown the percentage recovery from Ecteola of crude nuclear RNA extracts obtained by extracting the nuclear material of wound tissue at different stages of maturity. It can be seen that the Fractions IV of the crude RNA extracted from the nuclear material with
**TABLE VI**

RECOVERY OF RNA FROM THE EXTRACTS OF NUCLEAR MATERIAL ISOLATED AT DIFFERENT STAGES OF REGENERATION

<table>
<thead>
<tr>
<th>Days after wounding</th>
<th>Total crude nuclear RNA extracted from nuclear material *</th>
<th>Recovery of the total crude nuclear RNA from fractionation on Ecteola *</th>
<th>Percent recovery from fractionation on Ecteola</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>13.8</td>
<td>9.2</td>
<td>66</td>
</tr>
<tr>
<td>8</td>
<td>23.4</td>
<td>15.5</td>
<td>66</td>
</tr>
<tr>
<td>12</td>
<td>11.0</td>
<td>6.1</td>
<td>56</td>
</tr>
</tbody>
</table>

* In terms of O. D. Units per mg. tyrosine in the nuclear protein
TABLE VII

PERCENT DISTRIBUTION OF RNA IN THE FRACTIONS ISOLATED FROM THE NUCLEI OF WOUND TISSUE AT VARIOUS STAGES OF REGENERATION.

<table>
<thead>
<tr>
<th>Days after wounding</th>
<th>Extract</th>
<th>Fractions I</th>
<th>Fractions II</th>
<th>Fractions III</th>
<th>Fractions IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>neutral</td>
<td>21</td>
<td>21</td>
<td>12</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>weakly alkaline</td>
<td>16</td>
<td>55</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>strongly alkaline</td>
<td>21</td>
<td>35</td>
<td>11</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>neutral</td>
<td>2</td>
<td>3</td>
<td>11</td>
<td>84</td>
</tr>
<tr>
<td>8</td>
<td>weakly alkaline</td>
<td>73</td>
<td>18</td>
<td>9</td>
<td>--</td>
</tr>
<tr>
<td>strongly alkaline</td>
<td>55</td>
<td>20</td>
<td>5</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>neutral</td>
<td>10</td>
<td>15</td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>12</td>
<td>weakly alkaline</td>
<td>56</td>
<td>33</td>
<td>11</td>
<td>--</td>
</tr>
<tr>
<td>strongly alkaline</td>
<td>50</td>
<td>34</td>
<td>16</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>
nuetral\textsuperscript{solution} contain the largest part of the nuclear RNA. The amount of RNA in each fraction varies with the stage of development of the wound tissue and the extracting media. It is probable that each of these fractions contains a mixture of various kinds of RNA molecules. Many of the curves obtained in the RNA fractionation show considerable broadening of the descending limb, indicating incipient separation into further subfractions (Fig. 8).

**Size of the RNA Molecules in each Fraction**

The size of the molecules in the RNA fractions may be used as a criterion for distinguishing the fractions. The s-RNA molecules are known to be the smallest type of RNA (3). The r-RNA is supposed to have the largest molecular size (155). The size of m-RNA molecules varies between that of s-RNA and r-RNA. The crude nuclear RNA extracts obtained from the nuclear material of the wound tissue may be expected to contain all three types of RNA.

The possibility of some small degree of fragmentation of RNA molecules during the process of isolation and fractionation should not be overlooked. Precautions were taken to have the least possible degradation of the nuclear RNA molecules during varies procedures to which they were subjected. Thus, the extraction was carried out at 0\textdegree C. At this temperature the least possible degradation would be
expected. The fractionation was carried out at room temperature (at about 25 °C.). Although Ecteola fractions were stored in the cold as soon as they were collected, most of these fractions were exposed to this temperature for 6-10 hours. Thus there is a possibility that some of the fractions (particularly the Fractions IV which were eluted with 1.0 M LiCl in 0.05 M KOH) may contain some partially degraded RNA molecules.

The fractions eluted from Ecteola may be expected to be mixtures of RNA. With respect to the concentration of the salt used to elute each RNA fraction, the approximate size of the molecules in these fractions can be deduced. Previous work has shown that the RNA fractions which are eluted from Ecteola with high concentrations of salt solution (1 M) may have the largest molecular size (15, 141). This portion of the RNA may be considered to be mostly r-RNA. It accounts for most of the nuclear or cellular RNA (15, 62, 110, 136, 141). There are reports which indicate that these nuclear RNA fractions eluted with concentrated salt solutions (and having high sedimentation coefficients) possess some m-RNA activity (62, 156). The Fractions IV of the nuclear RNA of the regenerating wound tissue were eluted from Ecteola with 1.0 M LiCl in 0.05 M KOH. It could not be eluted from the column with lower concentrations of LiCl in neutral buffer. The largest part of the total crude
nuclear RNA extracted from nuclear material of wound tissue is found in these fractions (Table VII). It may be reasonably assumed that the Fractions IV of the nuclear RNA of regenerating wound tissue eluted from Ecteola consist largely of the nuclear r-RNA.

In another experiment, the nuclear RNA from the wound tissue, harvested 8 days after wounding, was fractionated. All of the RNA in Fraction 8 - 8.6 - IV and Fraction 8 - KOH - IV had molecular weights over 300,000. At least half of the RNA in Fraction 8 - 7.2 - IV had a molecular weight over 300,000 (Table VIII). This would indicate that the Fraction 8 - 7.2 - IV may contain different sizes of RNA molecules. The smaller molecules may have arisen from slight degradation of larger molecules. However, Fraction IV may be considered to contain mostly nuclear r-RNA.

Soluble-RNA has the lowest molecular weight of all the different types of RNA. For this reason, the molecules of s-RNA may be expected to appear at the beginning of the "fractionation profile" (Fig. 8). Chromatography of Fractions I on various Bio-Gel columns indicated that about 70-80% of the RNA in these fractions have a molecular weight of not more than 30,000; the remainder consist of RNA molecules with a molecular weight of at least 100,000. Therefore, the Fractions I may be considered to include all the s-RNA molecules with some
**TABLE VIII**

**PERCENT RECOVERY OF FRACTIONS IV OF NUCLEAR RNA FROM BIO-GEL COLUMNS.**

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Bio-Gel P-30</th>
<th>Bio-Gel P-100</th>
<th>Bio-Gel P-200</th>
<th>Bio-Gel P-300</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 - 7.2 - IV</td>
<td>86</td>
<td>44</td>
<td>54</td>
<td>61</td>
</tr>
<tr>
<td>8 - 8.6 - IV</td>
<td>110</td>
<td>104</td>
<td>94</td>
<td>114</td>
</tr>
<tr>
<td>8 - KOH - IV</td>
<td>113</td>
<td>136</td>
<td>141</td>
<td>128</td>
</tr>
</tbody>
</table>
admixture of the lower molecular weight m-RNA molecules.

The m-RNA molecules have molecular sizes ranging between s-RNA and r-RNA. Rapidly labeled RNA with m-RNA properties was shown to have sedimentation constant in the 12 S region (67,121).

Herriman and Hunter (62) have fractionated nuclear RNA on methylated serum albumin column. They eluted the RNA with stepwise increasing concentrations of 0.2 M, 0.4 M, 0.6 M, 0.8 M, 1.0 M and 2.0 M NaCl. When the fractions were assayed for amino acid incorporating activity into proteins, the fraction eluted with 0.6 M salt was found to have the highest stimulatory activity. The RNA component eluted with 0.6 M NaCl was considered to contain most of the m-RNA molecules. It had a sedimentation coefficient somewhat less than that of the 16 S r-RNA.

The true m-RNA has been characterized as the 16 S component (29,62).

Fractions II and III were eluted from Ecteola with 0.4 M and 0.6 M LiCl respectively in 0.05 M Tris buffer at pH 7.2. Chromatography of these fractions on various Bio-Gel columns showed that these fractions were heterogeneous. No approximation of the molecular size could be derived from the recovery of RNA components in these sets of fractions from chromatography on Bio-Gel. However, considering the size of RNA molecules which might be expected to be eluted from Ecteola by dilute neutral LiCl (29,62,122), the Fractions II and Fractions III might be expected to consist principally of m-RNA.
Base Composition of the Nuclear RNA Fractions

The base composition is another criterion which may be applied to distinguish the RNA fractions. The Ecteola fractions obtained from the three crude extracts of nuclear material of wound tissue ostensibly contain only four fractions on the basis of the elution profile. (Fig. 8).

The base composition of the various nuclear RNA fractions was determined by paper chromatographic procedures (182). Tables IX, X, XI and XII show the relative base composition of Fractions I, II, III and IV respectively.

Approximate similarity in base composition of the Fractions IV (Table IX) leads one to suspect that these fractions contain essentially identical mixtures or large amounts of the same kind of RNA molecules. The slight variations in base composition from fraction to fraction indicate that these fractions are by no means a single entity. At least some m-RNA molecules would be expected to be associated with the r-RNA, as has been reported to be the case with the r-RNA in other tissues (62, 156).

Different kinds of proteins would be expected to be present in different types of cells. It follows that the m-RNA molecules produced in one type of cell would be, to some extent, different from that produced in other type of cells. However, there is no reason to expect a change in the mechanism of protein synthesis from one type of cell to another.
<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Adenine</th>
<th>Guanine</th>
<th>Uracil</th>
<th>Cytosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 - 7.2 - IV</td>
<td>1.0</td>
<td>2.5</td>
<td>1.0</td>
<td>1.9</td>
</tr>
<tr>
<td>5 - 8.6 - IV</td>
<td>1.0</td>
<td>2.1</td>
<td>0.9</td>
<td>1.6</td>
</tr>
<tr>
<td>5 - KOH - IV</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>8 - 7.2 - IV</td>
<td>1.0</td>
<td>2.6</td>
<td>1.4</td>
<td>2.1</td>
</tr>
<tr>
<td>8 - 8.6 - IV</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>8 - KOH - IV</td>
<td>1.0</td>
<td>2.0</td>
<td>1.3</td>
<td>1.6</td>
</tr>
<tr>
<td>12 - 7.2 - IV</td>
<td>1.0</td>
<td>2.8</td>
<td>1.5</td>
<td>2.5</td>
</tr>
<tr>
<td>12 - 8.6 - IV</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>12 - KOH - IV</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

*Eluted from Ecteola with 1.0 M LiCl in 0.05 M KOH*

**Including methyl cytosines**
### TABLE X

*THE RELATIVE BASE COMPOSITION OF FRACTIONS I OF NUCLEAR RNA AT DIFFERENT STAGES OF WOUND TISSUE FORMATION*

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Adenine</th>
<th>Guanine</th>
<th>Uracil</th>
<th>Cytosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 - 7.2 - I</td>
<td>1.0</td>
<td>1.3</td>
<td>0.6</td>
<td>0.2</td>
</tr>
<tr>
<td>5 - 8.6 - I</td>
<td>1.0</td>
<td>1.3</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>5 - KOH - I</td>
<td>1.0</td>
<td>1.3</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>6 - 7.2 - I</td>
<td>1.0</td>
<td>1.7</td>
<td>1.8</td>
<td>0.7</td>
</tr>
<tr>
<td>6 - 8.6 - I</td>
<td>1.0</td>
<td>1.1</td>
<td>0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>6 - KOH - I</td>
<td>1.0</td>
<td>2.3</td>
<td>1.8</td>
<td>3.0</td>
</tr>
<tr>
<td>12 - 7.2 - I</td>
<td>1.0</td>
<td>1.2</td>
<td>3.1</td>
<td>2.0</td>
</tr>
<tr>
<td>12 - 8.6 - I</td>
<td>1.0</td>
<td>0.5</td>
<td>1.1</td>
<td>0.6</td>
</tr>
<tr>
<td>12 - KOH - I</td>
<td>1.0</td>
<td>1.4</td>
<td>1.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Eluted from Ecteola with 0.2 M LiCl in 0.05 M Tris buffer

**Including methyl cytosines
### TABLE XI

**THE RELATIVE BASE COMPOSITION OF FRACTIONS II OF NUCLEAR RNA AT DIFFERENT STAGES OF WOUND TISSUE FORMATION**

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Adenine</th>
<th>Guanine</th>
<th>Uracil</th>
<th>Cytosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 - 7.2 - II</td>
<td>1.0</td>
<td>0.9</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>5 - 8.6 - II</td>
<td>1.0</td>
<td>0.9</td>
<td>0.6</td>
<td>0.2</td>
</tr>
<tr>
<td>5 - KOH - II</td>
<td>1.0</td>
<td>0.8</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>8 - 7.2 - II</td>
<td>1.0</td>
<td>2.1</td>
<td>1.4</td>
<td>2.0</td>
</tr>
<tr>
<td>8 - 8.6 - II</td>
<td>1.0</td>
<td>0.6</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>8 - KOH - II</td>
<td>1.0</td>
<td>1.1</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>12 - 7.2 - II</td>
<td>1.0</td>
<td>2.5</td>
<td>1.7</td>
<td>1.8</td>
</tr>
<tr>
<td>12 - 8.6 - II</td>
<td>1.0</td>
<td>0.7</td>
<td>1.2</td>
<td>0.4</td>
</tr>
<tr>
<td>12 - KOH - II</td>
<td>1.0</td>
<td>0.7</td>
<td>1.3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* Eluted from Ecteola with 0.4 M LiCl in 0.5 M Tris buffer

** Including methyl cytosines
### Table XII

**The Relative Base Composition of Fractions III of Nuclear RNA at Different Stages of Wound Tissue Formation**

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Adenine</th>
<th>Guanine</th>
<th>Uracil</th>
<th>Cytosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 - 7.2 - III</td>
<td>1.0</td>
<td>2.0</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>5 - 8.6 - III</td>
<td>1.0</td>
<td>0.9</td>
<td>1.1</td>
<td>0.4</td>
</tr>
<tr>
<td>5 - KOH - III</td>
<td>1.0</td>
<td>0.7</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>8 - 7.2 - III</td>
<td>1.0</td>
<td>2.5</td>
<td>1.3</td>
<td>2.4</td>
</tr>
<tr>
<td>8 - 8.6 - III</td>
<td>1.0</td>
<td>1.1</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>8 - KOH - III</td>
<td>1.0</td>
<td>1.7</td>
<td>2.6</td>
<td>2.4</td>
</tr>
<tr>
<td>12 - 7.2 - III</td>
<td>1.0</td>
<td>2.6</td>
<td>1.6</td>
<td>2.5</td>
</tr>
<tr>
<td>12 - 8.6 - III</td>
<td>1.0</td>
<td>2.7</td>
<td>5.7</td>
<td>2.3</td>
</tr>
<tr>
<td>12 - KOH - III</td>
<td>1.0</td>
<td>2.7</td>
<td>5.3</td>
<td>2.9</td>
</tr>
</tbody>
</table>

*Eluted from Ecteola with 6.6 M LiCl in 0.05 M Tris buffer*

**Including methyl cytosines**
For the same reasons, it may be expected that the r-RNA molecules in most cells of an organism are similar. The data in Table IX supports this idea. The data show that the base composition of all Fractions IV remain practically unchanged despite the drastic change in cell population which occur in regenerating wound tissue between the 5th and 8th days after wounding (69). These results are in agreement with the findings that ribosomes from fibroblasts of various ages show no qualitative differences (86a). The similarity in base composition and the large amount of RNA in Fractions IV may indicate that these fractions contain mostly nuclear r-RNA.

The concentration of LiCl solution required to elute the Fractions I from Ecteola and the data on the recovery of these fractions from Bio-Gel suggest that these fractions contain all the s-RNA molecules. It may also contain fragments of RNA molecules which would be the degradation products of m-RNA or r-RNA. In spite of all the possible mixtures of RNA molecules which might be included in the Fractions I, the base composition of all the Fractions I on the 5th day after wounding is identical (Table X). The differences in base composition of Fractions I on the 8th and 12th days after wounding may be partly due to differences in the assortment of m-RNA molecules in these fractions.

Collagen contains a large porportion of glycine, proline and
hydroxyproline. The requirement of specific s-RNA molecules to transfer these amino acids into protein is increased when collagen synthesis increases. This requirement should become notable on the 8th and 12th days after wounding when fibroblasts, the cells producing the precursors of collagen, are the principal type of cells (38a). The presence of different specific s-RNA molecules may be a factor contributing to the difference in base composition of the Fractions I on the 8th and 12th days after wounding.

Fractions II and III contain most of the m-RNA molecules. The relative concentrations of the constituent bases in these RNA molecules in Fractions II and III are presented in Tables XI and XII. The Fractions II obtained from the nuclear RNA extracts of the 5th day seem to have similar base composition. These and several of the Fractions II obtained from wound tissue harvested on the 8th and 12th day of regeneration are peculiar in having a low cytosine content. Although not quite the same, there is a reasonable degree of similarity in base composition between the Fractions II at all stages of nuclear RNA formation in the wound tissue studied. This may be an indication that m-RNA molecules required for the synthesis of proteins common to all types of cells reside in this fraction.
From the base composition data in Table XII, it may be seen that Fractions III of the nuclear RNA isolated from the cellular nuclei of wound tissue, 5 days after wounding are different from each other and from the Fractions III isolated on the 8th and 12th days of regeneration. Fractions III obtained from the crude nuclear RNA extracted from nuclear concentrates by neutral solution on the 8th and 12th days after wounding have practically the same base composition. These two fractions represent about 70% (Fig. 8) of the total amount of RNA in Fractions III on these days. It may be possible to consider that RNA molecules in these fractions are the same. The possibility becomes more probable when it is known that the source of this RNA is from the nuclei of almost the same kind of cell population (69).

In general, Fractions III isolated from wound tissue on the 8th and 12th days of regeneration contain relatively more pyrimidine bases than purine bases. Two fractions isolated from the wound tissue on the 12th day have a very high uracil and low adenine content. No explanation can be given for the presence of this highly peculiar type of m-RNA. The purine to pyrimidine (Pu/Py) ratio of all the fractions III shows decreasing values as the wound tissue regeneration progresses. It has been reported that the Pu/Py ratio of the total RNA (50) and the nuclear RNA (63, 180) of wound tissue decreases with the regeneration. The
relative amounts of purines and pyrimidines of the possible code triplets would be expected to be found in m-RNA concerned with the biosynthesis of the precursor of collagen monomers were calculated (63). The Pu/Py ratio for rat skin collagen has been calculated to be approximately 0.82. The values shown in Table XIII probably may indicate that the RNA component in the regenerating wound tissue which accounts for the decrease in Pu/Py ratio may reside in Fractions III.

The results presented in this chapter were obtained from the wound tissue of a total of 59 rats; 20 each served on the 5th and 8th days and 19 on the 12th day after wounding. The crude nuclear RNA obtained from the wound tissue was fractionated on Ecteola columns. The Ecteola fractions were characterized according to the size of the RNA as nuclear s-RNA, m-RNA and r-RNA. Several of the Ecteola fractions have similar base composition. The similarities are thought to be due to the isolation of analogous RNA fractions from the same kind of cells on the 8th and 12th days. Fibroblasts are the most prevalent type of cell in the wound tissue at this time. The RNA molecules in Fractions III were considered to contain the RNA components which caused the observed decrease in Pu/Py ratio of the total RNA (50) and the nuclear RNA (180) of regenerating wound tissue.
TABLE XIII

THE PURINE TO PYRIMIDINE RATIO OF FRACTIONS III
ISOLATED FROM NUCLEAR MATERIAL OF WOUND TISSUE AT DIFFERENT DAYS OF REGENERATION

<table>
<thead>
<tr>
<th>Days after wounding</th>
<th>Purine to pyrimidine ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fractions III*</td>
</tr>
<tr>
<td>5</td>
<td>1.53</td>
</tr>
<tr>
<td>8</td>
<td>0.81</td>
</tr>
<tr>
<td>12</td>
<td>0.60</td>
</tr>
</tbody>
</table>

* Average of the three fractions obtained from the neutral, weakly alkaline, and strongly alkaline extracts of the nuclear material.
CHAPTER VII

FORMATION OF NUCLEAR RNA IN WOUND TISSUE

The relative rate of formation of RNA fractions may be used as a criterion for their characterization. The studies on the rate of formation and turnover of the nuclear RNA fractions are described in this chapter.

Incorporation of radioactive precursors into nuclear RNA of wound tissue would be expected to label RNA molecules which are rapidly formed in this tissue. The labeling experiments were carried out at the time when collagen formation in wound tissue is thought to be high (69, 177, 179). At this time (7-10 days after wounding), the RNA content of this tissue is at the highest level (69, 177, 179). It has been shown that the incorporation and turnover of P in nuclear RNA (which may be considered to be a measure of RNA formation and utilization), is parallel to the deposition of collagen in wound tissue (51, 179). Studies on the incorporation of labeled nucleosides in systems which are known to be actively producing collagen indicate that the uptake of the label into nuclear RNA is highest within one hour after the administration (37, 42).

The rate of formation and turnover of various nuclear RNA fractions were measured by following the rate of uptake of H-uridine.
Groups of 21 rats which had been wounded 8 days previously were given 3 115 curies of H-uridine subcutaneously. At intervals of 10, 20, 35 and 55 minutes thereafter, wound tissue samples were harvested from the rats in different groups. Nuclear RNA was isolated and fractionated according to the previous sections. The specific activity of the fractions was then measured.

The relative amounts of RNA in various fractions obtained from the nuclear material of wound tissue in this experiment is presented in Table XIV. The results appear to be in agreement with those of the previous experiment (Fig. 8), in that the major portion of the RNA was recovered in Fractions IV of the crude RNA extracted with the neutral solution from nuclear material. The relative amount of RNA in each fraction of this experiment seems to vary from the previous experiment even though the same experimental conditions were used. This could be due to the different degree of fragmentation of RNA molecules in each experiment. However, there seems to be a general correlation between the recovery of nuclear RNA of wound tissue in both experiments.

Relatively small changes have been noticed in the base composition of the fractions obtained in the two experiments. Considering the technique used for the isolation and fractionation of nuclear RNA in these experiments, minor changes in base composition may be anticipated.
### TABLE XIV

**RELATIVE AMOUNTS OF NUCLEAR RNA, RECOVERED FROM ECTEOLA, FROM REGENERATING WOUND TISSUE**

*ON THE 8th DAY AFTER WOUNDING*

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Crude Nuclear RNA extracted from nuclear material of wound tissue with</th>
<th>Neutral solution</th>
<th>Weakly alkaline solution</th>
<th>Strongly alkaline solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>+</td>
<td>1.6 - 0.6</td>
<td>0.5 - 0.5</td>
<td>----</td>
</tr>
<tr>
<td>II</td>
<td>+</td>
<td>3.3 - 0.9</td>
<td>1.1 - 0.4</td>
<td>1.2 - 0.5</td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>1.1 - 0.3</td>
<td>0.3 - 0.2</td>
<td>0.6 - 0.2</td>
</tr>
<tr>
<td>IV</td>
<td>+</td>
<td>10.8 - 2.1</td>
<td>1.1 - 0.2</td>
<td>1.1 - 0.4</td>
</tr>
</tbody>
</table>

*The values are expressed in terms of O.D. Units per mg. tyrosine in nuclear extract.*
However, Fractions IV obtained in these experiments have about the same base composition as those in the previous experiment (Table XV).

The Fractions IV, as previously pointed out, may be expected to be mostly r-RNA. The similarity of the base composition of the Fractions IV in both experiments may be due to the fact that there would be less chance to elute degradation products of other RNA moieties into these fractions. Still there may be some difference in the proportion of the amount of RNA components which constitute Fractions IV.

3

The rate of incorporation of H-uridine into the RNA of the various nuclear RNA Fractions IV is shown in Fig. 9. On the basis of specific activity, the rate of formation of the Fractions IV from the weakly alkaline extract of the nuclear material appears to be considerable greater than that of neutral and strongly alkaline extracts (Fig. 9). On the same basis, the rate of uptake of labeled uridine by Fractions IV from neutral and strongly alkaline extracts seems to be about the same.

The total amount of counts per minute is found to be maximal in the Fractions IV of the neutral extract (Fig. 10). This would indicate that the turnover of Fractions IV from the neutral extract is much more massive than those from the other extracts. In view of the finding that nuclear r-RNA of brain showed stimulatory effect in amino acid incorporation into proteins (62) due to the presence of m-RNA molecules which
<table>
<thead>
<tr>
<th>Bases</th>
<th>Expt. in Chapter IV</th>
<th>Expt. in Chapter V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Guanine</td>
<td>2.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Uracil</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Cytosine</td>
<td>1.6</td>
<td>1.9</td>
</tr>
</tbody>
</table>
Fig. 9: The rate of formation of Fractions IV of nuclear RNA of wound tissues of rats on the 8th day of wounding. Data is presented in terms of specific activity (cpm/μ moles uridine) of H-uridine. Fractions IV is eluted from Ecteola with 1.0 M LiCl in 0.65 M KOH. Data indicated by solid circles represent the Fraction obtained from the neutral extract of the wound tissue nuclear material; half-open circles, from the weakly alkaline extract; open circles from the strongly alkaline extract.
Fig. 10: The incorporation of H-uridine into Fractions IV of nuclear RNA of wound tissue on the 8th day after wounding. Data is presented in terms of counts per minute versus minutes after the subcutaneous administration of labeled uridine. Fraction IV is eluted from Ecteola with 1 M LiCl in 0.05 KOH. Symbols as in Fig. 9.
Fig. 11: The Rate of formation of Fractions I of nuclear RNA of wound tissue on the 8th day after wounding. Data is presented in terms of specific activity (cpm/μ moles uridine) versus minutes after the administration of labeled uridine. Fraction I is eluted from Ecteola with 0.2 M LiCl in 0.05 M Tris buffer. Symbols as in Fig. 9.
Fig. 12: The rate of formation of Fractions II of nuclear RNA of wound tissue on the 8th day after wounding. Data is presented in terms of specific activity (cpm/μ moles uridine) versus minutes after the subcutaneous administration of labeled uridine. Fraction II is eluted from Ecteola with 0.4 M LiCl in 0.05 M Tris buffer. Symbols as in Fig. 9.
Fig. 13: Rate of formation of Fractions III of nuclear RNA of wound tissue on the 8th day after wounding. Data is presented in terms of specific activity (cpm/μ moles uridine) versus minutes after the administration of labeled uridine. Fraction III is eluted from Ecteola with 0.6 M LiCl in 0.05 M Tris buffer. Symbols as in Fig. 9.
held the ribosomal clusters (polyribosomes) together (11, 57, 98), part of the radioactivity of Fractions IV may be accounted for by contamination with m-RNA. The rapid turnover of the nuclear r-RNA suggests the transport of the RNA molecules in these fractions into the cytoplasm within about an hour after the administration of labeled nucleosides as indicated in the work from other laboratories (37, 42).

As previously indicated, the elution properties and recovery from Bio-Gel columns suggest that the RNA molecules in Fractions I consist mostly of nuclear s-RNA. In Fig. 11 is shown the rate of uptake and turnover of Fractions I obtained from nuclear material of wound tissue 8 days after wounding. The data indicate that the RNA molecules in these fractions are formed only to a small extent and that these molecules turn over rapidly. These properties of the nuclear RNA Fractions I of the wound tissue are in agreement with the known properties of s-RNA in the cell.

Although m-RNA activity has been reported to be present in all nuclear RNA fractions isolated (62, 156), according to the elution pattern most of the m-RNA may be expected to be found in the Fractions II and III. The rate of formation of the RNA in Fractions II and III is presented in Fig. 12 and Fig. 13. It can be seen that the RNA molecules in Fractions III are formed and turned over much more rapidly than
those in Fractions II. Since the amount of H-uridine detected at any
given time represent the sum of both the rate of formation and the rate
of turnover, the actual rate of formation of Fractions III is probably
even greater than indicated in the graph.

The Fractions II contain more RNA than the Fractions III.

However, significantly more label was incorporated into the RNA molecules
of the Fractions III. This would signify that Fractions III contain more
rapidly formed m-RNA molecules than Fractions III. Herriman et al. (62)
have shown that a nuclear RNA fraction from brain tissue, corresponding
to Fractions III, is the most active of all the nuclear RNA fractions
in incorporating amino acids into protein. However, in view of the find-
ings of Herriman et al. (62) it may be considered that the Fractions III
of the nuclear RNA of wound tissue contain more rapidly formed m-RNA
molecules than Fractions II.

Fractions III from the neutral extract of the nuclear RNA is
formed and turned over most rapidly. The RNA in this fraction is
formed at least twice as fast as that from weakly and strongly alkaline
extracts and about four times or more as rapidly as in any of the Fractions
II. The rapid formation of RNA in this fraction may be taken as pre-
sumptive evidence that there is a requirement for large amounts of
some component of m-RNA in the wound tissue formation. In the wound
tissue, the formation of cellular protein as well as the formation of collagen are taking place. At the stage of regeneration studied, it has been reported that the DNA content and hence the number of cells of wound tissue begin to decline and cellular protein formation is expected to be low, (69, 177, 179). This would infer a decreasing requirement for m-RNA.

Specific m-RNA molecules are needed for the synthesis of cellular protein and collagen precursors. Most of the m-RNA formed in the nucleus of wound tissue cells on the 8th day after wounding is contained in Fractions II and Fractions III. It is hard to say which fraction contains more "collagen m-RNA." Such information can be obtained only by studying the stimulatory activity of the RNA in each fraction in the incorporation of amino acids into cell-free systems or purified cellular systems which normally do not produce collagen. However, since large amounts of collagen are being formed and deposited in the wound tissue at this stage of regeneration, it is highly suggestive that m-RNA molecules which are being formed rapidly, such as in Fractions III from the neutral extract, may be involved in the synthesis of monomeric precursors of collagen.
CHAPTER VIII

SUMMARY AND CONCLUSIONS

What Was Done!

Regenerating wound tissue on the 5th, 8th and 12th days after wounding, obtained from female rats, was homogenized and the nuclear material was isolated from the homogenate by differential centrifugation. The tyrosine content of the nuclear material was measured as a basis for comparing the amount of nuclear RNA. The RNA was isolated from phenolic suspension by stepwise extractions with neutral, weakly alkaline and strongly alkaline solutions (epichlorohydrin-triethanolamine derivative of cellulose).

The crude RNA extracts were further fractionated on Ecteola. Each of the crude RNA extracts were eluted with stepwise increasing concentrations of LiCl in neutral buffer, and finally with a high concentration of LiCl in strongly alkaline solution.

The nuclear RNA fractions, obtained from Ecteola, were hydrolyzed and the bases separated by paper chromatography. The four bases were individually extracted from the paper and measured spectrophotometrically.
The RNA fractions recovered from Ecteola were chromatographed on various Bio-Gel columns and the approximate molecular size calculated.

The rate of formation and turnover of the various nuclear RNA fractions of wound tissue were measured by following the rate of uptake of $^3\text{H}$-uridine into these fractions. After measuring the radioactivity, the uridine content of the RNA fractions was determined by paper chromatography. The specific activity of the RNA fractions were calculated.

What Was Found!

On the basis of nuclear tyrosine content, the yield of nuclear RNA was at the highest level on the 8th day of regeneration. Relatively more RNA was obtained in the neutral extract of the nuclear material than in the weakly or strongly alkaline extract.

Each crude nuclear extract could be fractioned into 3-4 fractions on Ecteola. The percentage of RNA in each fraction varies depending on the maturity of the wound tissue and extracting media. It is highly probable that each of these Ecteola fractions contains a mixture of several kinds of RNA moieties.

On the basis of elution characteristics and approximate molecular size, the nuclear RNA fractions containing s-RNA, m-RNA and r-RNA
were identified. The nuclear r-RNA fractions contained more RNA at all stages of regeneration studied.

The base composition of all the fractions reportedly containing the nuclear r-RNA, were approximately the same. Similarities in base composition were also observed between several other fractions.

The two sets of fractions, which contained the nuclear m-RNA molecules, were found to be formed faster than those fractions which were supposed to contain nuclear s-RNA or r-RNA. The m-RNA molecules in one set of fractions were formed and turned over many times faster than those of the other set of m-RNA fractions.

What the Findings May Mean!

The highest concentration of nuclear RNA on the 8th day may be attributed to the large amounts of collagen formed and deposited in the wound tissue cells at this time. Most of the m-RNA molecules produced in the nuclei of wound tissue cells are isolated into two sets of fractions. Relatively, the amount of m-RNA in one set of fractions is much smaller than in the other. The m-RNA in the small set of fractions has significantly high rate of formation and turnover. This may be taken as an indication that the m-RNA molecules in the small set of fractions are required in large amounts for the formation of wound tissue. Since large amounts of collagen are being formed in the wound tissue at this stage of regeneration,
it may be suggested that the m-RNA molecules in these sets of fractions, which are rapidly formed, are involved in the synthesis of the precursors of collagen.
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APPENDIX I

The diet was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, and had the following composition:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn Starch</td>
<td>70%</td>
</tr>
<tr>
<td>Alphacel (cellulose)</td>
<td>15%</td>
</tr>
<tr>
<td>Vegetable Oil</td>
<td>10%</td>
</tr>
<tr>
<td>Salt Mixture U.S.P. XIV</td>
<td>4%</td>
</tr>
<tr>
<td>Cod Liver Oil</td>
<td>1%</td>
</tr>
</tbody>
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The Salt Mixture U.S.P. XIV contained the following ingredients:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cupric Sulfate</td>
<td>0.48 gm.</td>
</tr>
<tr>
<td>Ferric Ammonium Citrate</td>
<td>94.33 gm.</td>
</tr>
<tr>
<td>Manganese Sulfate</td>
<td>1.24 gm.</td>
</tr>
<tr>
<td>Ammonium Alum</td>
<td>0.57 gm.</td>
</tr>
<tr>
<td>Potassium Iodide</td>
<td>0.25 gm.</td>
</tr>
<tr>
<td>Sodium Fluoride</td>
<td>3.13 gm.</td>
</tr>
<tr>
<td>Calcium Carbonate</td>
<td>6.86%</td>
</tr>
<tr>
<td>Calcium Citrate</td>
<td>30.83%</td>
</tr>
<tr>
<td>Calcium Biphosphate</td>
<td>11.28%</td>
</tr>
<tr>
<td>Magnesium Carbonate</td>
<td>3.52%</td>
</tr>
<tr>
<td>Magnesium Sulfate</td>
<td>3.83%</td>
</tr>
<tr>
<td>Ingredient</td>
<td>Percentage</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>12.47%</td>
</tr>
<tr>
<td>Dibasic Potassium Phosphate</td>
<td>21.88%</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>7.71%</td>
</tr>
</tbody>
</table>

The diet also contained the fat soluble Vitamins A and D and it was supplemented with B Complex Vitamins.
APPENDIX II

A. Folin-Ciocalteu Reagent: A mixture consisting of 100 gm. of sodium tungstate, 25 gm. of sodium molybdate, 700 gm. of water, 50 ml. of 85% phosphoric acid and 100 ml. of concentrated HCl was refluxed gently for 10 hours. After cooling 150 gm. of lithium sulfate, 50 ml. of water and few drops of liquid bromine were added. The mixture was boiled for 15 minutes without condenser to remove the excess bromine. It was cooled, diluted to 1.0 l and filtered through glass wool.

B. Carbonate-Copper Solution was prepared by mixing 50 ml. of 2% Na\textsubscript{2}CO\textsubscript{3} and 1 ml. of 1/2% CuSO\textsubscript{4} solution prepared in 1% sodium tartrate.

C. Scintillation Fluid - 4 g. of POP and 100 mg. of POPOP per liter of solution in toluene.
APPENDIX III

LIST OF ABBREVIATIONS USED

The following abbreviations commonly used in biochemical literature have been used in this dissertation:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMP</td>
<td>adenosine-monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-tri-phosphate</td>
</tr>
<tr>
<td>PPI</td>
<td>inorganic pyrophosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>s-RNA</td>
<td>soluble ribonucleic acid</td>
</tr>
<tr>
<td>r-RNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>m-RNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>gly</td>
<td>glycine</td>
</tr>
<tr>
<td>pro</td>
<td>proline</td>
</tr>
<tr>
<td>hypro</td>
<td>hydroxyproline</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>S</td>
<td>sedimentation</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Ecteola</td>
<td>Epichlorohydrin-triethanolamine derivative of cellulose</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
</tbody>
</table>
ABSTRACT

Formation and deposition of collagen in the wound tissue of rats take place most vigorously about 7-10 days after wounding. The amount of RNA in the regenerating wound tissue also reaches the maximal level at this time. Further, RNA in the cellular nuclei is reported to be formed especially rapidly at this stage of regeneration. The nature of the relation between collagen formation and the nuclear RNA is unclear. The purpose of this investigation was to obtain further information about the nuclear RNA in wound tissue and its possible relationship to the synthesis of collagen.

Wound tissue at different stages of regeneration was homogenized and the nuclear material isolated by centrifugation. The nuclear RNA was extracted from phenolic suspensions of the nuclear material with neutral, weakly alkaline and strongly alkaline buffer solutions. The extracts were further fractionated on Ecteola columns by stepwise elution with increasing concentrations of lithium chloride. The approximate molecular size and base composition of the various nuclear RNA fractions were determined.

The largest amount of nuclear RNA could be isolated when the total cellular RNA was at the highest level. The base composition of the nuclear RNA fractions, with the highest molecular weight (supposedly corresponding to ribosomal-RNA), was essentially the same at all the stages.
regeneration. The similarity in base composition of these fractions was found even when the type of cells found in the granulation tissue had changed drastically.

The fractions which were considered to consist primarily of m-RNA had a different base composition when isolated from wound tissue with almost no fibroblasts (in the early stages of regeneration) as compared to those from wound tissue in which fibroblasts were the most prevalent type of cell (in the later stages of regeneration). A gradual decrease in the purine to pyrimidine ratio of these nuclear RNA fractions was observed as the regeneration progressed.

The rate of formation and turnover of nuclear RNA in wound tissue were measured by following the uptake of tritiated uridine into the various RNA fractions. The labeling experiments were carried out on the 8th day of regeneration, when fibroblastic proliferation and deposition of collagen in the wound tissue were considered to be high. The ribosomal RNA was found to be formed relatively slowly and to accumulate in the nucleus. Slow turnover of r-RNA, presumably by the movement from the nucleus to the cytoplasm, takes place.

The m-RNA fractions were synthesized and turned over considerably more rapidly than any of the s-RNA or r-RNA fractions. One m-RNA fraction, representing about 5% of the total RNA isolated from the nucleus was formed extremely rapidly---at least four times as fast as the next
most rapidly formed m-RNA fraction. The rapid formation of RNA molecules in this fraction, at the time when rapid formation of collagen is taking place in the regenerating wound tissue suggests a requirement of some m-RNA component of this fraction for the synthesis of this protein.
APPROVAL SHEET

The dissertation submitted by Thomas C. Thachet has been read and approved by five members of the faculty of the Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY.

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Date

Martin B. Williamson
Signature of advisor