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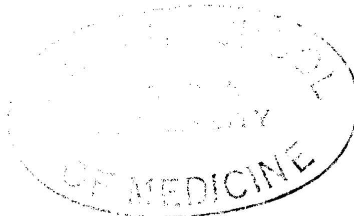
CHANGES IN NUCLEAR RNA DURING WOUND TISSUE REGENERATION

by

Eleanore Margaret Hertel



A Thesis Submitted to the Faculty of the Graduate School

of Loyola University on Partial Fulfillment of

the Requirements for the Degree of

MASTER OF SCIENCE

February

1964

LIFE

Eleanore Margaret Hertel was born in Erie, Pennsylvania, on June 1, 1939. She was graduated from Harbor Creek High School, Harbor Creek, Pennsylvania, in June, 1957.

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She is a member of Kappa Gamma Pi and of the Albertus Magnus Guild.

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CHAPTER I

THE CHARACTERISTICS OF COLLAGEN

The phenomenon of wound healing in past ages has been shrouded by an aura of mystery and superstition; it is only in modern times that any semblance of scientific study has been applied to the phenomena involved in an attempt to discover the mechanisms which cause the outward manifestations of tissue regeneration.

Direct microscopic analysis of regenerating wound tissue reveals a network of collagen fibers imbedded in a gelatinous matrix; fibroblasts, the cells which produce collagen fiber precursors, can be seen to be scattered through the network. In the initial stages of regeneration, the tissue is predominately cellular. As these cells manufacture collagen precursors and fiber formation proceeds, the tissue becomes relatively more fibrous and less cellular, with a consequent decline in metabolic activity. Hence, it becomes clear that collagen formation and deposition reflect the process of tissue regeneration. With the refinement of a variety of new experimental and analytical techniques applicable to protein chemistry, new aspects of collagen structure, biosynthesis, and metabolism have been opened for study (36).

Amino Acid Composition

Collagen is a fibrous protein which makes up an extracellular network which serves to unify the area of the integument as well as to provide

support for larger cellular structures. The collagen molecule is characterized by a glycine content of the order of 30 - 35% of the total amino acid residues. Two unique amino acids are found in collagen: hydroxylysine is present to the extent of about 2% and hydroxyproline to the extent of about 8% (this may vary up to 20%, depending on the source of the collagen) (36). Hydroxyproline is unusual in that it is found primarily in collagen and a few plant proteins (28). It has been shown that hydroxyproline administered to animals orally or by injection is not incorporated into collagen (79); consequently, it has been postulated that the source of collagenous hydroxyproline is proline, which has been hydroxylated either immediately before or after incorporation into the collagen structure (27). Collagen is also rather unique in that it contains little tyrosine and methionine, while tryptophan and cystine are completely absent. The amino acid composition of rat skin collagen is summarized in Table I.

Studies on the amino acid sequence of collagen have been carried out by Grassman and his associates (26). Amino acid end-group analyses of peptides isolated from tryptic hydrolysates of gelatin revealed, in almost all cases, the presence of three N-terminal glycines, two C-terminal arginines, and one C-terminal glycine. The estimated molecular weights of these fragments suggested the presence of triple chain peptides, but it has not been ascertained whether this phenomena is an artifact of hydrolysis or not.

No systematic distribution of either glycine or imino acids could be established, since peptides of the sequence glycyl-glycine,

TABLE I

AMINO ACID COMPOSITION OF RAT SKIN COLLAGEN

Residues/100 Total Residues (66)

Amino Acid	Tropo-collagen	1 Subunit	2 Subunit
3-Hydroxyproline	0.09	0.10	0.00
4-Hydroxyproline	9.2	9.6	8.6
Aspartic Acid	4.6	4.6	4.4
Threonine	1.96	1.99	1.98
Serine	4.3	4.2	4.3
Glutamic Acid	7.1	7.4	6.6
Proline	12.1	12.9	11.3
Glycine	33.1	33.0	33.6
Alanine	10.6	11.2	10.2
Valine	2.40	1.96	3.20
Methionine	0.78	0.80	0.61
Isoleucine	1.08	0.64	1.61
Leucine	2.38	1.81	3.24
Tyrosine	0.24	0.21	0.24
Phenylalanine	1.13	1.16	1.01
Hydroxylysine	0.57	0.43	0.80
Lysine	2.81	3.04	2.24
Histidine	0.49	0.19	0.85
Arginine	5.1	4.9	5.1
Amide N	(4.1)	(4.2)	(4.3)

glycyl-X-glycine, and glycyl-5X-glycine (X = another amino acid residue) were identified by end-group analyses. The latter evidence is in contradiction to the molecular model of collagen proposed by Rich and Crick (71). (See discussion which follows.) Attempts were made to correlate regions of peptides containing predominately polar amino acids with dark band regions observed in collagen fibers under the electron microscope and regions of peptides containing mainly nonpolar amino acids with light bands under the same conditions. More evidence must be obtained before the theory can be unquestionably substantiated. (See Fig. 1).

Molecular Structure

The current concept of the basic collagen molecule is that of a relatively rigid rod 2900 Å in length and 16 Å in diameter, having a molecular weight of approximately 360,000 (9). This unit has been designated "tropocollagen" by Schmitt, et.al. (75). A considerable portion of the molecule, if not its entire length, consists of three polypeptide chains, each with a left-handed helical conformation, the three helices being wound about each other in a right-handed fashion to form a triple stranded rope-like structure (71).(See Fig. 2). This conformation requires that at least one-third of the amino acid residues in collagen be glycine, since only two-thirds of the available residue positions can be accommodated by other amino acids.

Piez, et.al., have represented tropocollagen as containing two types of subunits, $\alpha 1$ and $\alpha 2$; these are present in the proportion of two $\alpha 1$ to one $\alpha 2$. These subunits are polypeptide chains of similar size,

Fig. 1: Schematic Representation of Collagen Fiber Showing Dark and Light Bands as Observed under Electron Microscope. (26).

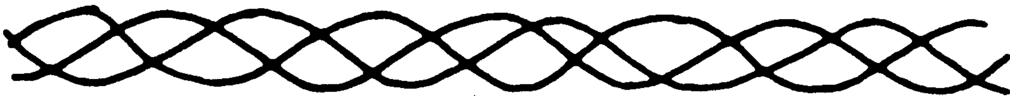


Fig. 2: Schematic Representation of Tropocollagen Molecule. (29).

but different amino acid composition. (See Table I). Under certain conditions, dimers of $\alpha 1$ and $\alpha 2$ can be isolated from collagen; these are designated $\beta 1$ and $\beta 2$. $\beta 1$ is a dimer consisting of an $\alpha 1$ unit crosslinked to an $\alpha 2$ unit, while $\beta 2$ is a dimer consisting of two $\alpha 1$ units crosslinked in a similar manner (66).

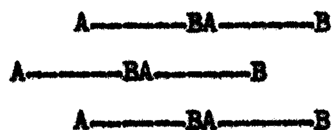
Bond Types Present in Tropocollagen

Due to the absence of cystine in collagen, no disulfide linkages are available for intra- or intermolecular binding. The three peptide chains of the tropocollagen molecule are linked primarily by intramolecular hydrogen bonds between the amide nitrogen of one peptide bond and carbonyl oxygen of the peptide link of the adjacent chain. Only every third peptide group may form such interchain bonds; consequently, other types of linkages have been sought. Gallop (23,24) has presented evidence for interchain ester and amide linkages and for γ-glutamyl peptide bonds in collagen. There is also the possibility that both the α-and Δ-carboxyl groups of aspartic acid are involved in intermolecular cross linking (8). The configuration of the peptide chains within the collagen molecule may give rise to inter- or intramolecular bonding; it is possible that more than one configuration of the peptide chain $\alpha 1$ is present at a given time and under certain conditions (66). Rich and Crick (71) have formulated two models for the collagen molecule which they have designated as Collagen I and Collagen II. In Collagen I, the hydroxyl groups of hydroxyproline are oriented inward, facilitating interchain bonding. In Collagen II, the same hydroxyl groups are oriented outward, favoring crosslinking between

molecules rather than between individual chains of a single molecule. Collagen II is sterically more stable and requires less distortion to accommodate amino acids other than glycine. Some physicochemical evidence is available to support both models (29).

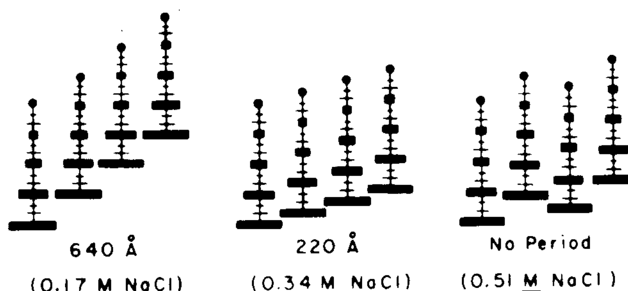
Polymerization of Tropocollagen

Tropocollagen molecules are asymmetric and associate together by their end regions, where the ends of the three polypeptide chains project by different amounts. The end regions of the peptides are rich in tyrosine, which is believed to be instrumental in binding the molecules together end to end (36). Under physiological conditions, the charge distribution on the surface of the asymmetric molecules is such as to make the most stable form of aggregate the one in which they overlap by one-fourth of their length, thereby giving rise to electron microscopically visible periods of 700 Å. If the terminal amino acid residues of a tropocollagen molecule are designated as A and B, collagen formed *in vivo* could be schematically represented in the following way:



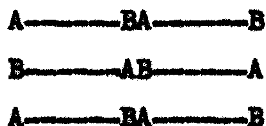
In vitro conditions such as an increase in ionic strength, or addition of high molecular weight substances such as glycoproteins, mucopolysaccharides, or nucleic acids, to the medium containing tropocollagen may cause alterations in the nature of the connection between molecules and/or changes in the periodicity of the fibers. The effect of an increase in ionic strength is illustrated in Fig. 3. "Fibrous long spacing"(FLS)

Fig. 3: Effect of Ionic Strength on Periodicity of Collagen Fibers.

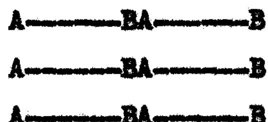


Each longitudinal unit schematically represents a tropocollagen particle with four major subdivisions evenly spaced along the length of the molecule. Each of the four subdivisions shows a further finer division into three parts. The degree to which the molecules are staggered as they line up will determine the nature of the period. At left, the longitudinal units present in 0.17 M NaCl are arranged in the "normal" period of 640 A. As the ionic strength of the NaCl solution is increased, the molecules arrange themselves in such a way as to decrease the length of the period. Finally, as ionic strengths such as that of 0.51 M NaCl are reached, the molecules arrange themselves in random fashion, and no period can be detected (see extreme right). (29).

collagen can be precipitated from acidic solutions of collagen by addition of high molecular weight substances; it is characterized by periods of up to 3000 Å and association of tropocollagen molecules in antiparallel array without overlap, as shown in the following:



Precipitation of collagen in the presence of ATP results in "segment long spacing" (SLS) collagen; it is characterized by association of the molecules in parallel array without overlap, as shown in the following:



Other _in vitro_ conditions have been observed to cause precipitation of two dimensional sheets and possibly even three dimensional lattice-like aggregates (32).

Soluble Collagens

Soluble collagens represent a group of biologically young collagens which can be brought into solution under certain conditions without loss of native structure. This is not to be confused with the dissolution of insoluble collagen in hot water resulting in gelatin formation. The latter is an irreversible denaturation process resulting in the destruction of the secondary structure of the protein.

Acid soluble collagen, or "procollagen", as it has been termed by Orekhovitch (64), is considered to be a type of young collagen newly formed and probably to be found on the outside of fibers or fibrils.

It is a non-homogeneous substance and is thought to exist in solid form in the tissues. It has been shown to have essentially the same amino acid composition as insoluble collagen (66). Precipitation with chondroitin sulfate *in vitro* gives rise to the formation of fibers by a process thought to be analogous to fiber formation *in vivo* (36).

Neutral salt soluble collagen is thought to be a precursor of both acid soluble and insoluble collagens. It is soluble in the same solutions as acid soluble collagen and would be extracted with this fraction were it not first removed by extraction with neutral sodium chloride solutions (31). It has been shown in guinea pigs that dietary restrictions and/or vitamin C deprivation cause a reduction in the neutral salt soluble collagen (33, 30). Green, et.al., have shown that a portion of the extractable neutral salt soluble fraction is associated with intracellular microsomes, but more specific estimates of the intra- and extracellular distribution cannot be made at this time (27).

Aging of collagen appears to decrease its solubility, but the exact nature of the change is not yet understood.

Collagen Fiber Formation

Collagen may be observed at the tissue level in the form of extracellular fibers ranging in size from 0.2 microns to diameters visible to the naked eye. Minute examination of mammalian skin collagen fibers reveals the presence of discrete parallel or interlacing unbranched fibrils varying in diameter from 100 Å to 0.2 microns. It is generally agreed that small fibrils are formed initially; these then thicken as the tissue

matures. The maturation process may be a matter of days (in vitro conditions in tissue culture) or a matter of weeks and months (normal in vivo conditions). In studies with chick embryos, Fitton-Jackson (48) has described a decrease in the amount of interfibrillary material which accompanies the increase in fibril size.

In the early stages of fibril formation, particularly in wounds, the arrangement of the direction of the fibrils appears to be random; however, it is apparent that fibril orientation must be regular during the later stages of development. Electron micrographs have revealed a certain regularity both in the spacing between fibrils and in the band alignment in parallel fibrils (48, 87, 89, 90). Fibrils have usually been observed to run in the same direction, although Karrer (51) has described fibrils lying in opposite directions. The mechanism by which fibril orientation is controlled remains quite obscure.

The site of collagen fiber formation has not been definitely elucidated at the present time. The present consensus of opinion is that the fibroblast synthesizes tropocollagen, which is then secreted into the extracellular spaces where it aggregates with other similar units to form a fibril. There is a possibility that, under certain circumstances, polymerization of tropocollagen within the cell could occur; the thin fibrils formed could then be extruded to the extracellular space where they could increase in size by accretion (29). There is an alternative possibility that the bulk of fibrils appear de novo in the extracellular spaces by aggregation of tropocollagen units in a process similar to that observed in vitro. There is evidence to support the aggregation of collagen

molecules either at the cell surface or at a distance from it (10, 47). What appear to be collagen fibrils have been observed under certain conditions within fibroblasts in avian tissues (47). It is possible that these fibrils may have been precipitated within the cytoplasm, or that extracellularly formed fibrils may have been encompassed by fibroblasts in transit through tissue. Alternatively, extracellular matrix containing collagen in solution may have been engulfed and fibrils polymerized within the vacuole inside the cell. Chapman (14) has lately proposed that collagen in mammals may be deposited in the extracellular space by shedding of cytoplasm.

CHAPTER II

BIOSYNTHESIS OF PROTEINS

A knowledge of the structure and composition of collagen fibers is required prior to considering the mechanisms involved in the formation of this protein. The formation of collagen may be anticipated to involve a mechanism similar to that required for the synthesis of other proteins, even though this protein has unique properties and composition. Collagen formation may be considered at two levels: at the level of formation of the polypeptide chains $\alpha 1$ and $\alpha 2$, and at the level of polymerization of these units into tropocollagen and collagen fibers. The latter has been considered in the previous section. This chapter will be concerned with the mechanism of formation of the polypeptide chains.

The Interrelationship of DNA and RNA to Protein Synthesis

DNA molecules consist of long double stranded chains of polydeoxynucleotides arranged in the form of helices and bound to basic nuclear proteins. The sequence of the deoxynucleotides in the DNA is believed to be of paramount importance in that it determines the information contained within the molecule. In the presence of the enzyme, DNA-polymerase, and the appropriate deoxynucleoside triphosphates, DNA helices produce complimentary RNA chains which have been designated "messenger"RNA. The messenger RNA strands, containing the information stored in DNA, then move from the nucleus to the ribosomes in the cytoplasm. These

discrete particles, consisting of approximately 50% RNA and 50% protein, are considered to be the site of most cellular protein synthesis. In the cytoplasm, free amino acids react with ATP through the intervention of the appropriate activating enzymes to form aminoacyl adenylates. These compounds remain bound to the enzyme surface; through the intervention of the same enzymes, they are transferred to soluble or transfer RNA and thus transported to the ribosomes. The transfer RNA-aminoacyl complexes are aligned on the ribosomes as dictated by messenger RNA, and peptide bond formation takes place. The newly-formed protein and the intact transfer RNA are then released from the microsomal surface; the messenger RNA utilized is believed to be inactivated in the process. The process is summarized in Fig. 4 (63). Many of the details of the processes described have yet to be clarified. Current experimental evidence will be cited as each step is examined in greater detail.

Formation of DNA

X-ray diffraction studies led Watson and Crick (88) in 1953 to conclude that DNA consists of two helical strands of deoxyribonucleotides wound together and held in position by hydrogen bonds between complementary base pairs, the bases including adenine, guanine, cytosine, and thymine. This postulation received further support some years later when Warner et.al. (86) observed that synthetic polynucleotides, such as polyadenylic acid, interacted in solution to form double-stranded helical structures similar to that proposed for DNA. The theory was further substantiated by Kornberg (53, 7) in 1960 with the isolation of an enzyme which would

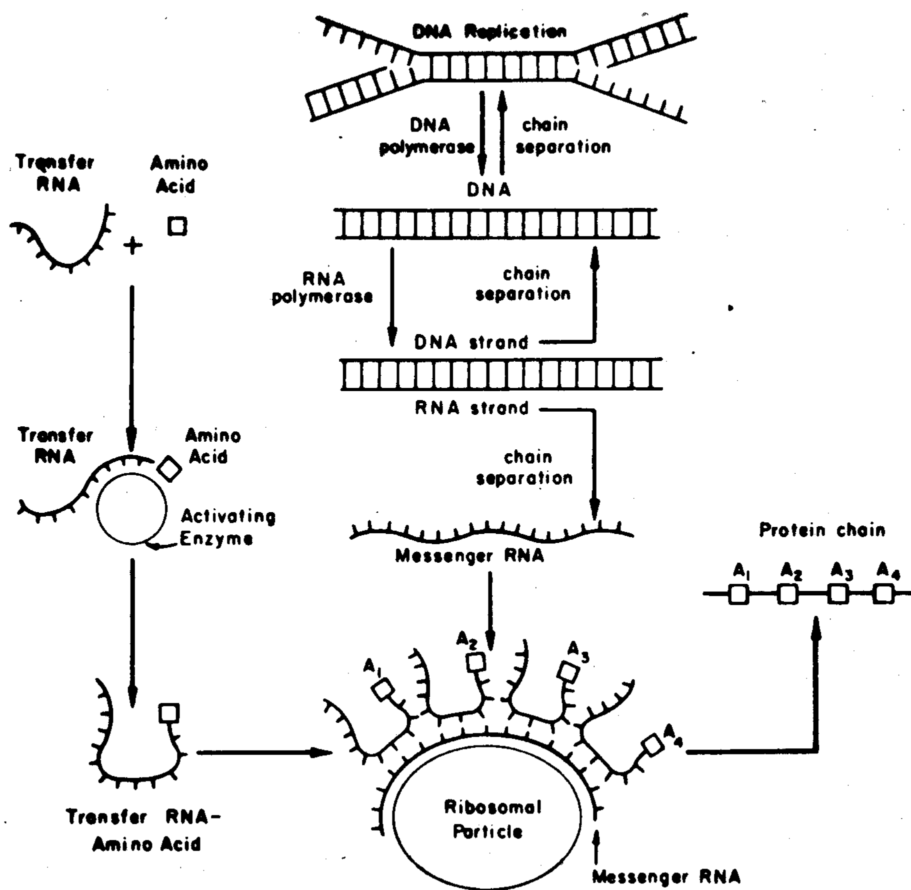


Fig. 4: Schematic outline of nucleic acid function. The ladder-like figures represent double-stranded nucleic acids and the bases are represented by the short cross lines. See p. 13 and 14 for a complete discussion of the various figures (63).

synthesize DNA from deoxyribonucleoside triphosphates with liberation of inorganic pyrophosphate. The enzyme, termed DNA-polymerase, requires the presence of nucleoside triphosphates and a DNA primer for activity. It is thought that DNA polymerase has the ability to separate the chains of the DNA molecule and to organize the proper complementary nucleoside triphosphates along each single strand; polymerization then occurs with the production of two identical molecules.

Role of DNA in Protein Synthesis

It is well known that DNA does not participate directly in cytoplasmic protein synthesis (13). It was originally thought that DNA played a more direct role in nuclear protein synthesis, but more recent work has shown this not to be the case. When the enzyme, deoxyribonuclease, is added to an *in vitro* preparation of nuclei, DNA is hydrolyzed and amino acid incorporation comes to a halt. However, amino acid incorporation may again be stimulated by addition of a DNA supplement, or partially denatured DNA, or RNA, or a variety of polyanionic compounds (4). Deoxyribonuclease has been shown by the same workers to inhibit oxidative phosphorylation in the nucleus. It has been suggested that amino acid incorporation *per se* is not damaged, but that the impaired energy mechanism will no longer support incorporation.

Significance of Amino Acid Activation

In a series of experiments patterned after the work of Maas and Novelli (58) on the formation of a pantoyl-adenylate by reaction of

pantothenic acid and ATP with consequent release of pyrophosphate, Hoagland et.al. (39) showed that a slow exchange of pyrophosphate and ATP in soluble extracts of rat liver was strongly stimulated by a mixture of natural amino acids. Furthermore, in the presence of amino acids and hydroxylamine, hydroxamates were formed e.g. leucyl-hydroxamate was identified. The reactions can be summarized as follows:

Equation I: Enzyme + ATP + amino acid $\rightleftharpoons$ Enzyme-AMP amino acid + PP

Equation II: Enzyme-AMP amino acid + NH_2OH $\longrightarrow$

Enzyme + AMP + amino acid-NHOH

De Moss et. al. (17), by incubating chemically synthesized leucyladenylate and inorganic pyrophosphate in bacterial extracts, were able to demonstrate the reverse of Equation I. Thus, the direct activation of amino acids was established.

Continued experimentation revealed that when total amino acid concentration in the incubation mixture was kept constant, ATP-pyrophosphate exchange was stimulated by an increase in the number of amino acids present (13). This seemed to indicate that the individual amino acids did not compete for a single enzyme, but rather that each reacted with a different enzyme. At present, several amino acid-specific activating enzymes have been isolated and purified from various tissues. These enzymes probably contain an -SH group at the active center, since activity is inhibited by p-chloromercuribenzoate and protected by reduced glutathione (1, 16).

It has been observed that Equation II can be made to proceed as written only in the presence of high concentrations of hydroxylamine. This is due

to the fact that the aminoacyl adenylates remain tightly bound to the activating enzymes. This serves as a protective measure, since at physiological pH, free aminoacyl adenylates would form peptide bonds in random fashion via a non-enzymic process (13).

Refinements in subcellular fractionation procedures led to the isolation by Zamecnik et.al. (40, 52) of the activating enzymes. When the pH of the supernatant remaining after separation of nuclei, mitochondria, and microsomes, was adjusted to 5.0, a precipitate formed. When redissolved and added to a synthetic amino acid incorporating system consisting of an energy source capable of regenerating ATP, microsomal particles, GTP, and amino acids, the release of pyrophosphate from ATP was observed, indicating that the precipitate must have contained the activating enzymes.

Similar synthetic amino acid incorporating systems have been utilized in an attempt to determine the mechanism by which the activated amino acids are transferred to the microsomal particles. Hoagland et.al. (41) found that the "pH 5" enzyme fraction contained about 5% RNA; furthermore, when the preparation was incubated with ATP and C^{14}-leucine, the isolated RNA became labeled. When this C^{14}-leucine-RNA was added to microsomal particles, the leucine could eventually be isolated in the microsomal proteins. The RNA which mediates the transfer of amino acids from the adenylate to the microsomal RNA has been termed "transfer RNA".

Transfer RNA

Various attempts to isolate the transfer RNA present in the supernatant remaining after subcellular fractionation have established the fact that

there is a specific soluble RNA for each individual amino acid. The RNA molecules are estimated to contain between 50 and 100 nucleotide residues and to have a molecular weight in the range of 10,000 to 50,000 (2, 77). It has been observed that when crude transfer RNA is loaded with leucine, at maximum it can bind only about one leucine residue per two thousand nucleotide residues (25, 38). Since there are twenty amino acids, it would seem that the estimate of 100 nucleotide residues per molecule is reasonably accurate. Also, it would seem that each transfer RNA molecule transfers only one amino acid residue.

The transfer RNAs differ from other cellular RNAs in that they are soluble in M NaCl and in that they contain appreciable amounts of pseudo-uracil nucleotide. The terminal end of the polynucleotide chain consists of two cytidylic acid residues in the pentultimate positions and one adenylic acid residue at the terminus. Fixation of amino acids is possible only when these three residues are intact (37). The terminal adenylic acid residue is attached via its 5' phosphate group to the 3' position of the preceeding cytidylic acid residue; it is believed that the amino acid attaches at the 2' or 3' position of the adenine nucleotide (13). It has been established that the transfer of a free amino acid to adenylic acid and thence to transfer RNA is mediated by the same activating enzyme (56). However, very little is understood of the mechanism by which the amino acid is passed from transfer RNA to the microsomal particle. It has been postulated that the helical structure of transfer RNA allows for three unpaired bases at one end of the molecule; these bases may recognize certain triplet base groups present in messenger RNA at the microsomal site and thereby permit

alignment of the amino acids so that peptide bond formation can occur. A schematic representation of a transfer RNA molecule with an attached amino acid is shown in Fig. 5.

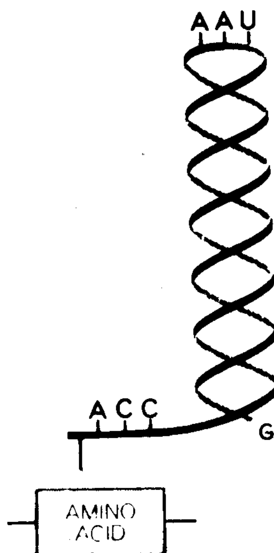
Microsomal RNA

Microsomal RNA and its functions is probably the least understood of the various RNA species. It was originally thought that microsomal RNA served as a template for protein synthesis. With refinement in experimental techniques, it has become evident that this is not necessarily the case. Many workers have observed that the composition of microsomal RNA is similar in a number of bacterial species, yet the variations in the corresponding DNAs are appreciable. Volkin, using incubation mixtures of *E. coli* infected with "T-even" phages (85), observed that a short-lived RNA having the characteristics of phage messenger RNA was rapidly formed after infection. Phage proteins were also subsequently formed. Examination of the microsomal RNA present revealed that no phage microsomal RNA was formed; hence, it could be concluded that microsomal RNA did not function as a template for protein synthesis in this instance.

Microsomal RNA is estimated to comprise 80-90% of the total cellular RNA; it exists, bound to protein, as discrete ribonucleoprotein particles, contributing about 50% of the particle weight.

Tissieres et.al. (80), using microsomal particles isolated from *E. coli*, has observed that the intact 70 S ribosome is actually composed of two subunits having sedimentation coefficients of 50 S and 30 S respectively. Kurland (54) has shown that the 50 S subunit consists either of one RNA

Fig. 5: Schematic Diagram of Transfer RNA



The terminal CCA sequence of nucleotides must be intact in order for an amino acid to be attached to the molecule. The unpaired bases at the upper end of the diagram are believed to be functional in recognizing the specific messenger RNA which codes for the attached amino acid.

molecule having a molecular weight of 1,120,000 and sedimentation coefficient of 23 S, or of two RNA molecules, each having a molecular weight of about 560,000 and a sedimentation coefficient of 16 S. The 30 S subunit consists of one of the latter molecules. Hence, each microsomal particle appears to consist of two molecules of high molecular weight RNA; a "large" one and a "small" one; in some cases, three "small" molecules have been reported (78). This observation probably holds true for other types of RNA.

It appears that the RNA in the microsomes consists of long continuous polynucleotide chains, made up of 1500 to 2000 (small component) and 4000 to 4500 (large component) nucleotide residues each. The concentration of magnesium in the medium is known to affect the state of aggregation of the molecules. In the presence of a high concentration of magnesium ions, there is the possibility that chains of 6000 polynucleotide residues may exist (78).

Messenger RNA

Messenger RNA is that type of RNA which carries information from the DNA in the nucleus to the extranuclear sites of protein synthesis. It is believed to be formed by the action of RNA polymerase in the presence of the four ribonucleoside triphosphates and a DNA primer. The DNA primer may be single or double stranded; however, certain types of RNA polymerases prefer the double stranded primer to the single stranded one, so that RNA formation is more efficient when the former is used (63). When double stranded DNA is utilized for messenger RNA production, there is evidence that both strands are duplicated, the

base composition of the RNA being the same as that of the DNA primer with uracil replacing thymine.

There is some evidence to support the theory that under *in vivo* conditions, RNA can replicate on only one DNA strand e.g. if the terminal end of one strand of a double stranded DNA molecule were bound in some way so as to prevent the action of RNA polymerase, then no messenger RNA could be formed from that strand. Presumably, it would be possible to release this bond without denaturing the double stranded helical structure, so that under *in vitro* conditions, both strands would be available for RNA synthesis (78, 96).

Messenger RNA is known to exist in very small amounts in cells e.g. less than 8% of the total RNA in bacteria (78). It is thought that messenger RNA molecules are variable in size, depending upon the nature of the protein to be synthesized. Various workers have reported molecular weights varying from 30,000 to 2,000,000, but some of these values may be artifacts resulting from partial degradation of the intact molecules during the isolation process (78).

Transfer of Information for Protein Synthesis

The recognition of messenger RNA as the carrier of information from DNA to the site of protein synthesis has aroused tremendous interest in the manner in which this information is stored in messenger RNA. As early as 1954, Gamow (95) observed that if a four-letter "alphabet" consisting of the nitrogenous bases A, G, C, and T (See appendix I) was to specify a twenty word "dictionary" corresponding to the twenty amino

acids, each code word would have to contain at least 3 bases. If each code word contained only two bases, only 4 x 4 or 16 words could be coded, whereas 3-base code words would allow 4 x 4 x 4 or 64 code words to be formed. The latter case would allow ample code words for the twenty amino acids, as well as numerous other code words which might code such information as "begin here" or "stop here". Recent experimentation by Nirenberg and Matthaei, and Ochoa and associates (62) has provided evidence for this theory.

An ingenious method for the study of the code words described above has been developed by Nirenberg and Matthaei (61). Cell-free extracts are prepared by crushing bacterial cells so as to release the cell sap containing DNA, messenger RNA, ribosomes, enzymes, and other soluble components. When provided with an energy source, these extracts will incorporate amino acids into protein. Incorporation can be followed by use of C^{14}-amino acids.

Addition of deoxyribonuclease to a cell-free system has been shown to have no effect on the initial rate of amino acid incorporation, but subsequent incorporation is greatly reduced. It would seem, then, that DNA serves as a template for messenger RNA synthesis, while messenger RNA in turn serves as a template for protein synthesis. Initially, when some messenger RNA is present, protein synthesis continues; when the supply is exhausted and no more can be made, protein synthesis stops.

In addition, it was further observed that messenger RNA from various sources will stimulate protein synthesis. Synthetic polynucleotides,

produced by the action of the enzyme polynucleotide phosphorylase on the appropriate mixture of nucleoside triphosphates, appears to have the same effect (62, 50).

By adding various synthetic polynucleotides to a cell-free amino acid synthesizing system, Nirenberg and Matthaei (62) have been able to observe the manner in which information is stored in messenger RNA. Using polyuridylic (poly U) acid as a synthetic messenger RNA, they were able to isolate a polypeptide consisting exclusively of phenylalanine residues. Ochoa and associates (55) extended this work by adding various combinations of other nucleoside triphosphates to poly U. The ratio of phenylalanine to other amino acids incorporated was observed to be approximately proportional to the expected occurence of various triplet permutations in random sequences formed by particular mixtures of nucleoside triphosphates. Thus, a mixture of nucleoside triphosphates in the proportion of 5U:1A, in the presence of polynucleotide phosphorylase, was expected to yield polynucleotides containing the following proportions of triplets: UUU=25; UUA=UAU=AUU=5; AAU=AUA=UAA=1; AAA=0.2. When these polynucleotides were added to a cell-free protein synthesizing system, polypeptides composed of phenylalanine or tyrosine or asparagine were isolated in the ratio of 25:5:1. Thus it appears that the code for phenylalanine is UUU; for tyrosine, UUA or UAU or AUU; for asparagine, AAU or AUA or UAA. The various triplet codes derived in this way for the twenty amino acids are summarized in Table II. It will be noted that the code appears to be degenerate i.e. in some cases, more than one triplet has been shown to cause the incorporation of an amino acid.

TABLE II

CODE DICTIONARY OF PURINE AND PYRIMIDINE BASES INVOLVED IN THE BIOSYNTHESIS OF PROTEINS (62)

Amino Acid	RNA Code Words			
Alanine	CCG	UCG*		
Arginine	CGC	AGA	UCG*	
Asparagine	ACA	AUA		
Aspartic Acid	GUA			
Cysteine	UUG#			
Glutamic Acid	GAA	AGU*		
Glutamine	ACA	AGA	AGU*	
Glycine	UGG	AGG		
Histidine	ACC			
Isoleucine	UAU	UAA		
Leucine	UUG	UUC	UUA	UUU•
Lysine	AAA	AAG"	AAU"	
Methionine	UGA*			
Phenylalanine	UUU			
Proline	CCC	CCU'	CCA'	CCG'
Serine	UCU	UCC	UCG	
Threonine	CAC	CAA		
Tryptophan	GGU			
Tyrosine	AUU			
Valine	UGU			

#Uncertain whether code is UUG of GGU.

*Need for U uncertain

•Codes preferentially for phenylalanine.

"Need for G and U uncertain

'Need for U, A,G uncertain.

Additional experimentation is necessary before it can be concluded with certainty that either the code is degenerate, or that the multiple triplets observed for some amino acids are actually due to artifacts inherent in the experimental technique.

Another type of experimentation, in which messenger RNA from tobacco mosaic virus is treated with nitrous acid, has shown that conversion of one purine to another, or of one pyrimidine to another, results in the substitution of one amino acid for another in the protein produced. Such changes are designated "transitions". Other changes involving the interchange of a purine with a pyrimidine have also been observed; these changes are known as "transversions" (50). Such experimentation has given rise to the concept of "the shared doublet" i.e. the suggestion that certain amino acids may be coded by triplets that have two nitrogenous bases in common. Hence, one amino acid may be substituted for another by a change of only one nitrogenous base in its particular messenger RNA code word. The significance of this concept becomes apparent when one considers that various inherited defects have been shown to be due to the substitution on one amino acid for another in a given protein e.g. sickle cell anemia. Continued experimentation in these areas will undoubtedly serve to elucidate some of the most fundamental concepts of biochemistry.

CHAPTER III

NUCLEIC ACIDS IN REGENERATING WOUND TISSUE

Various sources of regenerating tissue have been used in the study of nucleic acid metabolism during the regenerative process. Histochemical techniques and colorimetric analyses of the RNA and DNA content of the tissue have both been utilized; in spite of the diversity of both source material and methods of measurement, the results of investigations are rather consistent.

The RNA content of regenerating wound tissue has been shown to increase after infliction of the wound, reaching a maximum approximately 7-10 days later (34, 44). Hosoda (44), in addition, was able to demonstrate that uptake of P^{32} into RNA of regenerating wound tissue in mice increased to a maximum at the fourth day after wounding, declined slightly at the sixth day, and reached a new maximum at the eighth day before tapering off at the twelfth day after wounding. The initial maximum was attributed to the high RNA content of leucocytes which had infiltrated the wound area; this inflammatory reaction was observed histologically and shown to decline by the fifth or sixth day. The maximum on the eighth day coincides with the period of active fibroblastic proliferation and fiber formation; hence, it would seem that there is a definite relation between the progress of regeneration and RNA synthesis. These results are substantiated by the findings of Tsanev (82), who observed that RNA is conserved during periods of intense mitotic proliferation.

Tsanev has also observed a rapid disintegration of existing RNA immediately following trauma; it is believed that the disintegration products cause the accumulation of leucocytes at the wound site, thereby effecting a biological cleansing of the area and preparing a favorable environment for granulation tissue formation (81, 83). The observations of Palladina and Gudina (65) on the action of purine-containing substances supports this theory, as do histochemical studies made by Raekallio (68).

Several attempts have been made to elucidate the role of vitamins C and A (5, 73, 84) on nucleic acid metabolism in regenerating wound tissue. Rudas (73) observed that if guinea pigs were maintained on a scorbutic diet for 5 days prior to wounding, the RNA content of the wound tissue was decreased as compared to control values. This situation could be avoided by supplementing the scorbutigenic diet with vitamin C during the preoperative period. Administration of vitamin C after wounding to animals which had been maintained on a scorbutigenic diet previous to wounding did not cause RNA levels to return to normal. Tsanev (84) observed somewhat different results. While an impairment of collagen synthesis could be observed early in the regenerative process, he did not detect a decreased RNA content in the wound until two weeks after wounding. He postulated that the effect of vitamin C was not directly on RNA levels, but on oxidation-reduction processes of the cell. Any such impairment would be reflected in decreased levels of organic phosphorus compounds, thereby decreasing the RNA precursor pool and the high-energy phosphate compounds which provide most of the energy for protein synthesis.

Williamson and Guschlbauer have studied extensively the formation and turnover of nucleic acids during the regenerative process in rats (35, 92, 93, 94). They observed an increase in the RNA content of the wound tissue which reached a maximum at about 8 days after wounding. Determination of RNA-phosphorus which measures total RNA and of purine-bound ribose which measures only the purine content of RNA enabled the pyrimidine content of the RNA to be calculated by difference (91). The ratio of purines to pyrimidines was then calculated at intervals after wounding. In the early stages of wound healing, the ratio of purines to pyrimidines is about one, indicating that these components are present in equal amounts on a molar basis. In the later stages, there appears to be a preponderance of pyrimidines in the RNA, as indicated by a decreased purine : pyrimidine ratio. This may indicate that more than one specific type of RNA may be produced during the regenerative process. The fact that more than one type of protein, e.g. extracellular collagen and intracellular proteins, has been observed in the wound tissue lends credence to this theory.

Further studies by the same authors (35) on the types of protein produced in regenerating wound tissue have revealed that while the amount of collagen in the wound increases throughout the regenerative period, the concentration of intracellular proteins remains essentially constant. Also, a protein or peptide rich in sulfur-amino acids appears to be formed, paralleling the course of collagen formation. The requirement of sulfur-amino acids for the repair process has been widely recognized, but its exact significance remains obscure (91).

In measuring P^{32} incorporation *in vivo* into RNA associated with the nuclear, ribosomal, and soluble fractions of regenerating wound tissue in rats, Williamson and Guschlbauer (92) observed a change in the formation and turnover of nuclear RNA paralleling collagen formation in a manner analogous to that described for the sulfur-rich protein. It appears that several types of RNA are formed during the regenerative process. That type associated with the nuclear fraction is thought to be concerned with collagen formation, since both are formed slowly and cannot be detected in appreciable amounts until the repair process is fairly well advanced. By way of contrast, the RNA associated with the ribosomal fraction is a very large fraction of the total RNA, but appears to have a very rapid turnover during the early stages of wound healing. This occurs coincidently with the period of maximal cell proliferation in the wound tissue; hence, it seems probable that this type of RNA is associated with cellular protein synthesis. The soluble fraction seems to be very rich in RNA during the early phases of wound healing, but its turnover is very low as measured by P^{32} uptake. This may be due to the presence of oligonucleotides resulting from increased nuclease activity immediately after wounding (92). Later in the regenerative process, the amount of soluble RNA decreases, and the turnover rate becomes almost negligible.

Objectives of the Work Reported in This Thesis

The principal purpose of this investigation is a more detailed study of nuclear RNA than has previously been reported. According to the current theory of protein synthesis and to the results of the investigation of

amino acid incorporation into nuclear proteins (3, 43, 57, 70), it would seem that at least two groups of RNAs are present in cellular nuclei. One group is concerned with nuclear protein synthesis and consists of nuclear messenger RNA, nuclear ribosomal RNA, and nuclear transfer RNA. The other group is composed of the various messenger RNAs necessary for extranuclear protein synthesis. These may be further subdivided into those RNAs concerned with cellular protein synthesis and those concerned with collagen precursor biosynthesis. The latter classification has already been suggested by Williamson and Guschlbauer (94). It would seem, then, that analysis of both the content and composition of nuclear RNA during the regenerative process would yield valuable information regarding the nature of the RNAs present and their possible functions. Accordingly, three types of measurements will be made:

1. The determination of purine-bound ribose will be utilized as a measure of the RNA content of the nuclear fraction. Any changes in nuclear RNA levels during the regenerative process can then be detected. Measurement of total cellular RNA by Williamson and Guschlbauer (92) has revealed changes in RNA levels during wound healing; similar changes may or may not be observed in the nuclear fraction of wound tissue. There is the possibility that the changes in total cellular RNA observed may have been the result of changes occurring in one particular subcellular fraction; if this is the case and major changes in cellular RNA levels do not take place in the nuclear fraction, the results of this series of experiments may be expected to differ appreciably from the findings of Williamson and Guschlbauer.

The determination of purine-bound deoxyribose will be utilized as a measure of the nuclear material present in the preparations. Nuclear protein concentrations of the preparations will be utilized as a basis for calculation in comparing RNA and DNA levels in various preparations. e.g. Amounts of ribose and deoxyribose will be calculated per mg. nuclear protein.

2. Hydrolysis of the nuclear RNA to liberate the nitrogenous bases which will then be separated chromatographically. In view of the role of messenger RNA in the storage of information concerning protein synthesis, a comparison of the amounts of nitrogenous bases isolated and the amounts of nitrogenous bases calculated to be required to code for a protein of the amino acid composition of collagen might give an indication of the relative amount of nuclear RNA which is concerned with collagen biosynthesis at various intervals during the regenerative process.

3. Administration of various RNA precursors *in vivo* to determine the extent of incorporation of these precursors. The primary purpose of these experiments is to observe the incorporation characteristics of three guanine derivatives in an attempt to ascertain which would be the most feasible for use in studies on the rate of incorporation of RNA precursors into nuclear RNA.

The experimental procedures employed in the proposed investigation will be described in the following chapter.

CHAPTER IV

EXPERIMENTAL PROCEDURES AND METHODS

In the following experiments, female Sprague Dawley albino rats (200-225 gms.) were used. Wounds were made under ether anesthesia by excision of a four cm. circle of skin from the back down to the deep fascia as previously described (94). Wounds were swabbed with 95% ethanol as an antiseptic procedure. The wounded animals were kept in individual cages for periods of 5, 8, or 12 days after wounding on a limited stock diet. Water was permitted *ad libitum.* To collect the wound tissue, the animals were sacrificed by an overdose of ether and the tissue immediately removed and frozen in liquid nitrogen. The frozen tissue samples were stored in a deep freeze at -15° C. until they could be homogenized.

Since only a very small amount of nuclear material could be isolated from the tissue obtained from one animal, tissues from several animals were pooled at this point and crushed, while frozen, by repeated blows of a hammer on a steel pestle in a cold steel mortar. During this crushing process, the tissue was repeatedly treated with liquid nitrogen to ensure its being kept in a frozen and brittle state. The crushed tissue was then transferred to a chilled flask and suspended in cold 0.25 *M* sucrose containing 0.003 *M* Ca^{++} (approximately 1-3 gms. of tissue per 60 ml.) and shaken in the cold room for 3-4 hours (42, 76).

The suspended homogenized tissue was centrifuged at 600 rpm for 5 minutes at 2° C. Upon histological examination, the resulting precipitate

appeared to consist largely of shreds of insoluble collagen particles and was discarded. The supernatant was again centrifuged at 600 rpm for 10 minutes (II) and finally again at 1800 rpm for 20 minutes (III) at 2° C. Further fractionation yielded fractions which appeared to contain essentially no nuclei upon histologic examination. After separating the three fractions described, the remaining supernatant containing non-nuclear fragments was discarded.

The precipitates resulting from the centrifugation at 600 rpm (II) and 1800 rpm (III) were studied histologically in an attempt to identify the subcellular components. Smears were prepared from small amounts of the precipitates and stained with either hematoxylin and eosin (to differentiate nuclear and cytoplasmic material) or aniline blue (to identify the presence of collagen). Microscopic examination revealed these precipitates to consist of intact nuclei contaminated with significant amounts of small collagen fragments and some cytoplasmic debris. Extensive experimentation was conducted in an attempt to purify these fractions, with little or no success. However, use of the crude fractions without further purification could be justified for several reasons:

1. Collagen fragments would not interfere with the isolation or identification of nuclear RNA because the insoluble proteins would be precipitated previous to extraction of RNA.

2. The major amounts of RNA in the cell are associated with the nucleus, the microsomes, and with the soluble supernatant. Under the conditions of centrifugation 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 microsomes, etc.; consequently, no significant contamination by cytoplasmic RNA was to be expected. Microsomal RNA and soluble RNA, then, would not be expected to contribute appreciably to the contamination of the nuclear RNA present.

__Isolation of Nuclear RNA__

The RNA in the nuclear fraction of the regenerating wound tissue was isolated by a modification of the method of Schmidt and Thannhauser (74). Pricipitates (II) and (III) obtained by the above procedure were combined and suspended in 10.0 ml. of 7% perchloric acid and centrifuged in the cold at 3800 rpm for 20 minutes. The precipitate was washed with a small amount of distilled water, re-centrifuged, and the supernatant discarded. A 5.0 ml. portion of 0.3 __N__ KOH was added to the washed precipitate in the centrifuge tube. After suspending the precipitate in the alkali by brief mixing, the tube was incubated for one hour at 37° C. The suspension was immediately centrifuged in the cold at 3800 rpm for 10-15 minutes. The supernatant was decanted into a clean centrifuge tube and the precipitate, consisting mainly of insoluble proteins, was washed with distilled water and the washings added to the supernatant. The precipitate was discarded.

Acidification of the supernatant with 2.0 ml. of 2 __N__ perchloric acid resulted in the formation of a copius white precipitate consisting of DNA and nuclear proteins. The precipitate was collected by centrifugation for 15 minutes at 3800 rpm. The supernatant was decanted and the precipi-

tate washed with distilled water, the washings being added to the supernatant. The supernatant, containing mono- and polyribonucleotides, was filtered and made up to a volume of 25.0 ml., the final concentration of perchloric acid being 2 N. The precipitate was dissolved in 1 N NaOH and made to a volume of 10.0 ml. All samples were stored in the cold until the analytical procedures could be carried out.

Determination of Nuclear RNA

The RNA from the nuclear fraction of the wound tissue was measured by the method of Ceriotti (11). This technique is specific for the measurement of the color formed by the reaction of purine-bound ribose with orcinol.

The reaction of ribonucleotide samples with orcinol was found to give rise to a brown color which interfered with the measurement of the normal green color resulting from the orcinol-ribose reaction. The substance giving rise to this brown color could be destroyed by making the ribonucleotide aliquot alkaline to a final concentration of 0.1 N NaOH and incubating in a boiling water bath for 15 minutes prior to reaction with orcinol. This procedure does not appear to affect the ribose bound in the nucleotide (69).

The procedure consists of adding orcinol reagent to 5.0 ml. samples of ribonucleotide solution and to standard samples of the same volume containing 5.0 - 15.0 μ moles of D-ribose, followed by heating the mixture at 100° C. for 40 minutes. The orcinol reagent is prepared on the day of use by dissolving 100 mgs. of orcinol in 10 ml. of 0.04 M $CuCl_2$ (dissolved

in concentrated HCl) and diluting to 100 ml. with concentrated HCl. After cooling, the reaction mixtures were extracted for 10 minutes on a mechanical shaker with an equal volume of iso-amyl alcohol. One extraction appeared to remove essentially all of the color from the aqueous phase (45). The optical density of the alcoholic extract was measured at once at 660 millimicrons in a Klett-Summerson colorimeter. All determinations were made in triplicate. A typical standard curve is represented in Fig. 6.

Determination of DNA

The reaction of purine-bound D-2-deoxyribose with indole (12) was used to measure the DNA in the nuclear fraction. To a 4.0 ml. aliquot of DNA solution and to 4.0 ml. standards containing 3.0 to 15.0 μ moles of D-2-deoxyribose was added 2.0 ml. of concentrated HCl and 2.0 ml. of 0.04% aqueous indole. The reaction mixtures were heated in a boiling water bath for 10 minutes. Upon cooling, the reaction mixtures were extracted by shaking for 10 minutes with an equal volume of chloroform to remove extraneous color caused by reaction of proteins in the solution. Repeated extractions with chloroform have been advocated in the literature, but no significant change in the reading of the aqueous phase could be observed upon repeated extraction; consequently, only a single extraction was employed. The optical density of the aqueous phase was measured at 500 millimicrons in a Klett-Summerson colorimeter. All samples were measured in triplicate. A typical standard curve is represented in Fig. 7.

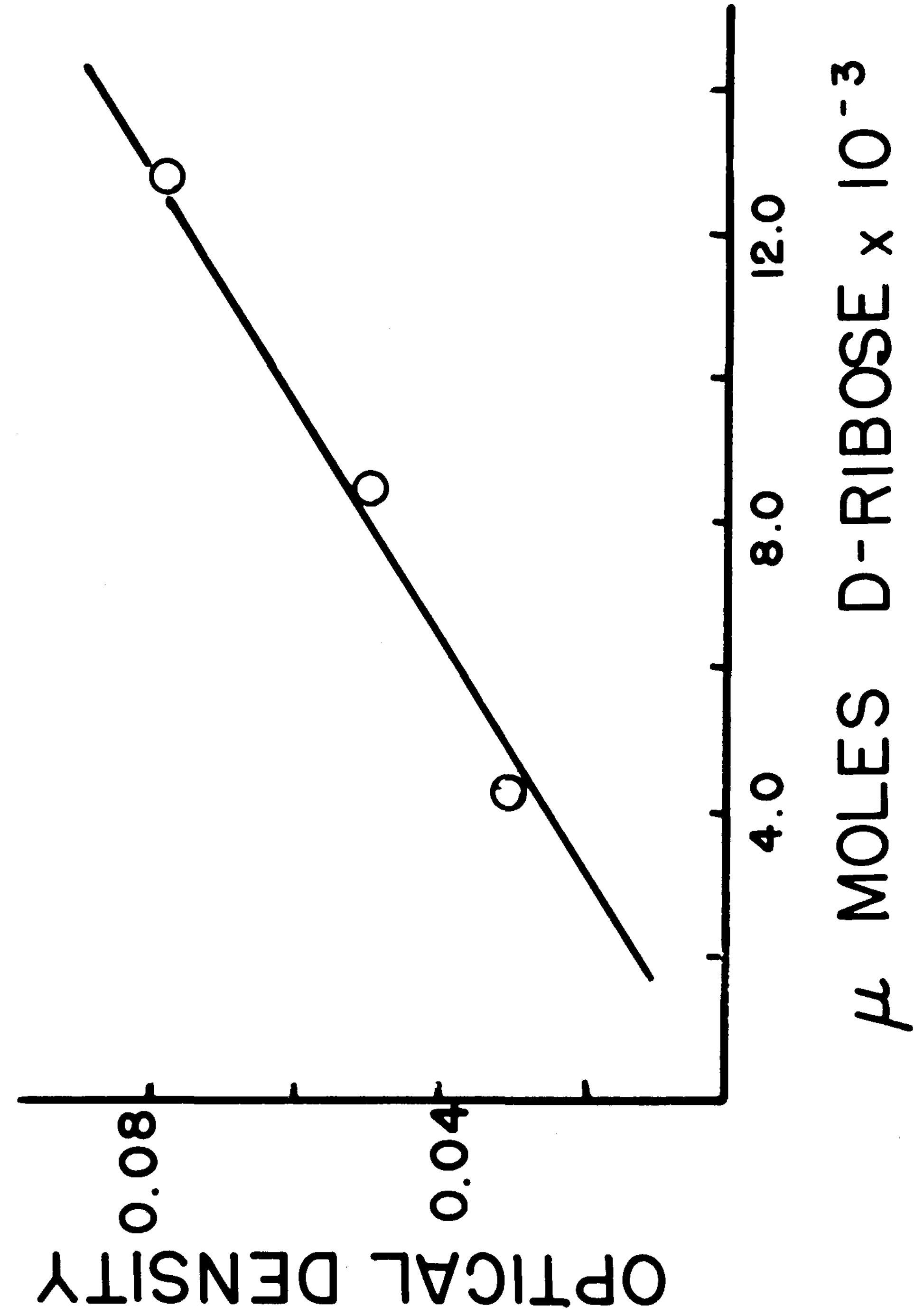


Fig. 6: Standard curve obtained by plotting optical density against the amount of D-ribose in each sample after determining the D-ribose content of the samples according to the method of Ceriotti(11) as described on p. 37. Since fresh reagents were prepared for each determination, a new standard curve consisting of three points was constructed for each determination.

Determination of Total Protein

The amount of protein in the samples was measured by comparison of tyrosine content of the nucleoproteins present in the DNA fraction with that of bovine plasma albumin by means of the reaction with Folin-Ciocalteu phenol reagent (22). Standard samples were prepared containing 0.0 - 2.5 mgs. bovine plasma albumin in a volume of 1.0 ml.; 1.0 ml. aliquots of the DNA fraction were also prepared. To each sample was added 1.0 ml. of Folin-Ciocalteu phenol reagent (diluted 1:3) and 2.0 ml. of 1.5 M Na_2CO_3. The reagents were mixed and the color was allowed to develop in the dark for 20 minutes. The tubes were read at 540 millimicrons in a Klett-Summerson colorimeter. A typical standard curve is represented in Fig. 8.

Chromatographic Separation of Nitrogenous Bases Present in RNA

The method described by Cohn (15) was used to separate the nitrogenous bases in the RNA isolated from the nuclei present in regenerating wound tissue. The cation exchange resin, Dowex 50 X8, was prepared by washing successively with 1 N NaOH, 1 N NaCl, and 2 N HCl interspersed with several rinsings in distilled water between each wash. After each suspension in distilled water, the resin was allowed to settle slowly so that there would be a separation of the large intact particles of the resin from "the fines". The latter, consisting of broken resin beads, settle very slowly and could easily be decanted. Inclusion of "fines" with the resin in a column tends to reduce the rate of flow through the column.

The prepared resin was suspended in distilled water and poured into

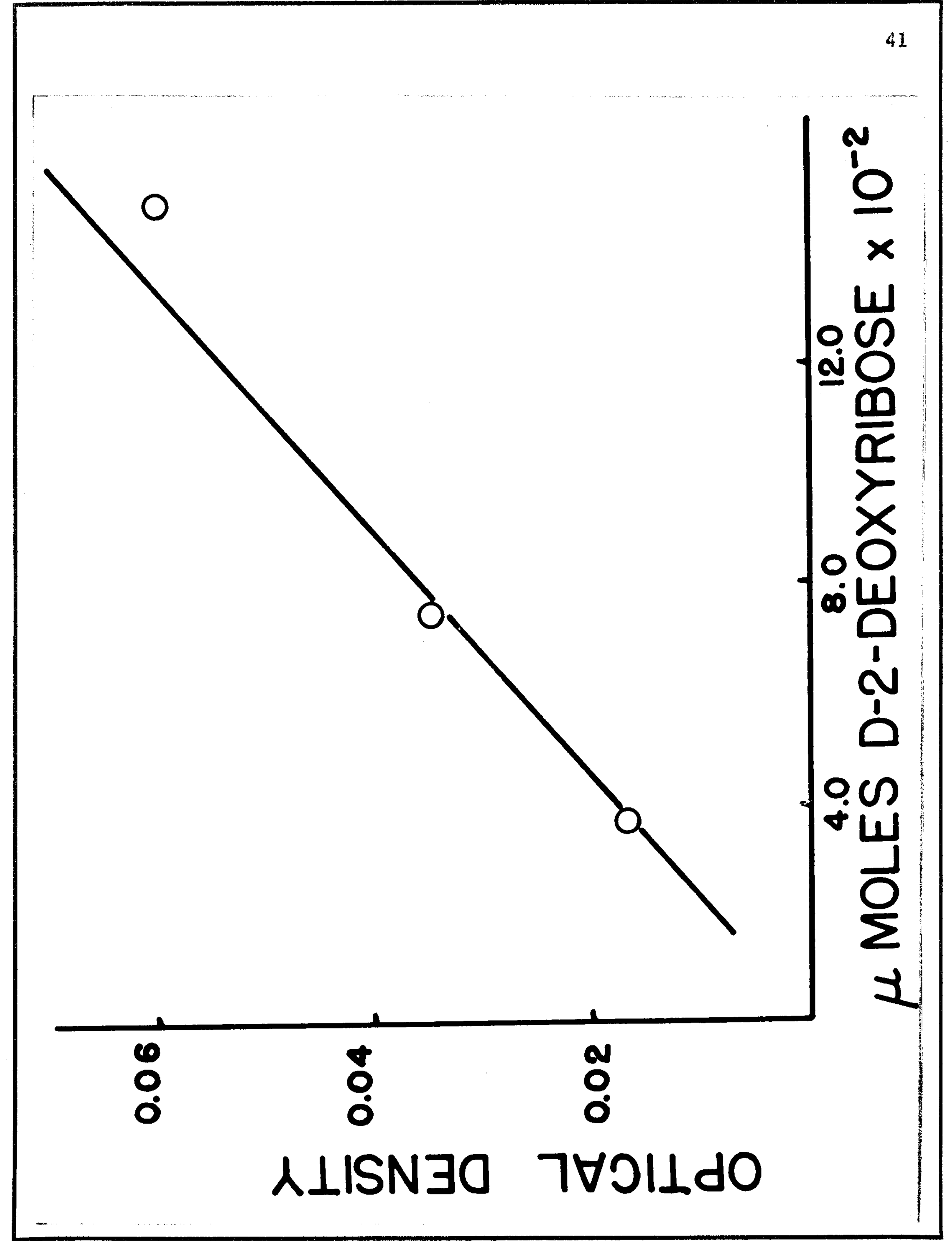


Fig. 7: Standard curve obtained by plotting optical density against amount of D-2-deoxyribose in each sample after determining the deoxyribose content of the samples according to the method of Ceriotti (12) as described on p. 38. A standard curve consisting of three points was constructed with every determination of experimental samples.

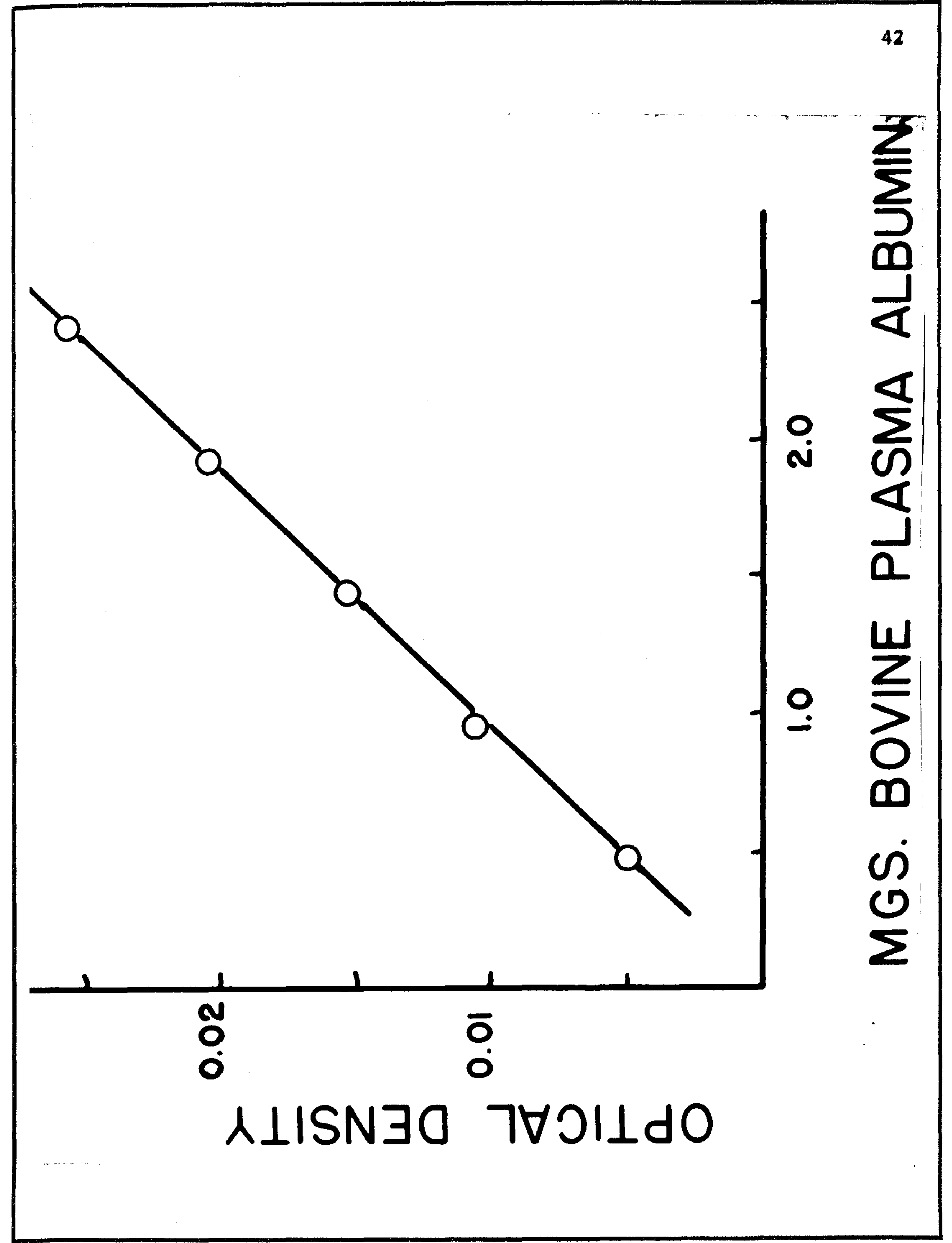


Fig. 8: Standard curve for Folin-Ciocalteu reaction. Samples of bovine plasma albumin were prepared in concentrations of 0.0 to 2.5 mgs./ml.; 2.0 ml. of 1.5 M Na_2CO_3 and 1.0 ml. of Folin-Ciocalteu reagent (diluted 1:3) were added. The mixture was shaken and the color allowed to develop for 20 minutes in the dark. Samples were read at 540 millimicrons and the optical density plotted against concentration of the sample as shown above.(22).

the column to be used for the chromatographic separation. This column had an inside diameter of 10 mm. and a resin length of 10 cm. The resin was washed with 2 N HCl for at least 30 minutes before use. The flow rate through the column could be regulated by means of a stopcock at the base of the column; it was usually maintained at about 2.0 - 2.5 ml. per minute. The same column was used for several separations; it was repoured only when air bubbles had become entrapped in the system. After the column had been used for a separation of the nitrogenous bases, the column was washed with water for at least 30 minutes and stored under water until its next use.

Solutions of the various nitrogenous bases containing 0.0 - 2.5 moles of base per ml. were used to calibrate the column. A 2.0 ml. aliquot of the standard solution was allowed to settle into the column and was then eluted with 2 N HCL. Fractions of 5.0 ml. were collected by means of a Vanguard fraction collector. Ultra-violet absorbing materials were detected by means of a Beckman DU spectrophotometer with an attached Photovolt recorder. All samples were read at 265 millimicrons. The areas under the peaks obtained by means of the recorder were estimated by planimetry and by triangulation. A standard curve was constructed for each base by plotting the area under the curve against the concentration of the solution. These standard curves are shown in Fig. 9.

Samples of the ribonucleotide solutions isolated from the nuclear fraction of the wound tissue were hydrolyzed in 2 N perchloric acid in sealed tubes for 3 hours in a 100° C. oven. Aliquots were placed on the column in a manner analogus to that described above. The column was eluted with 2 N HCl until approximately 600 ml. had been eluted.

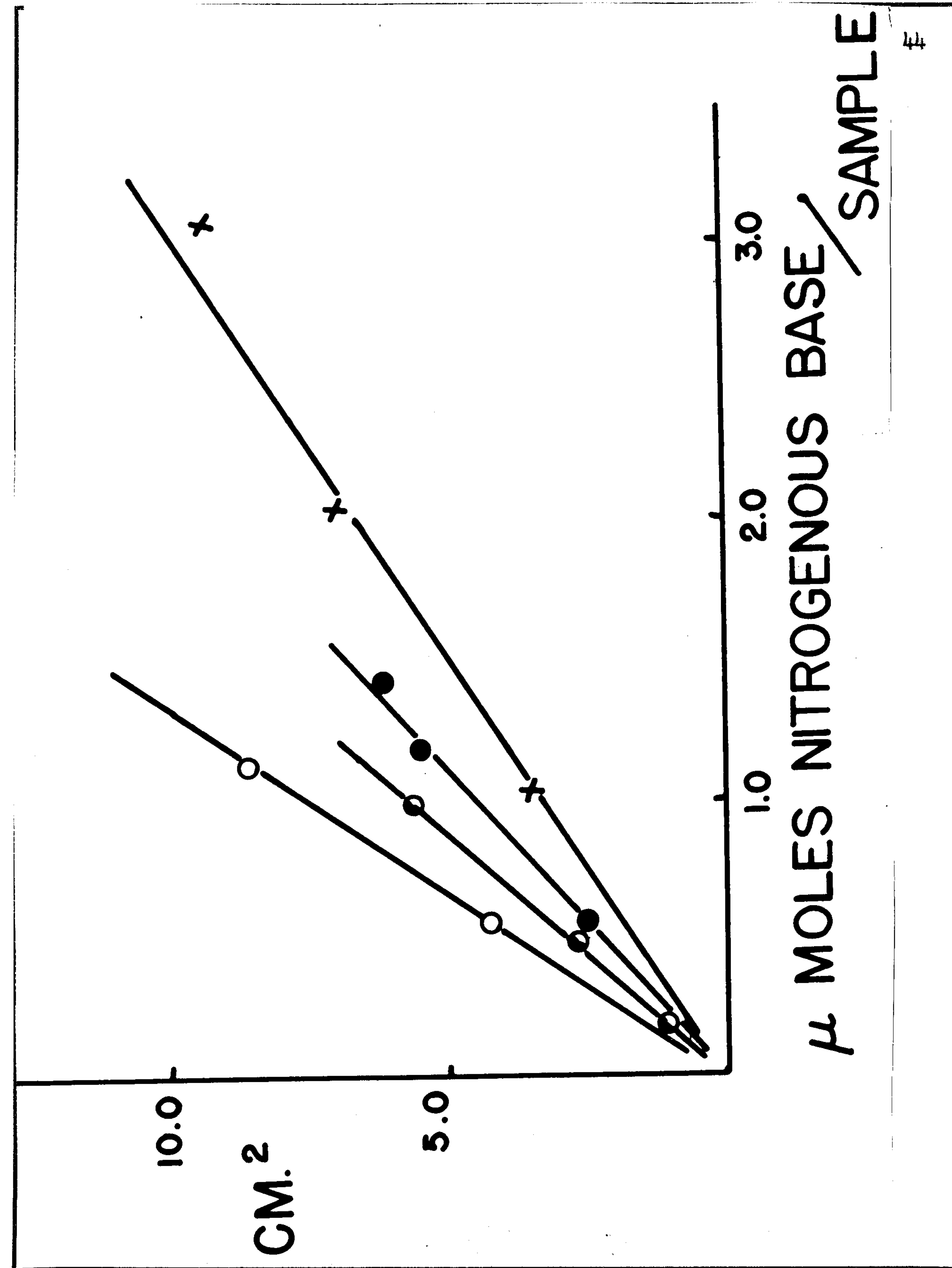


Fig. 9: Calibration of cation exchange column. Samples of the four nitrogenous bases known to be present in RNA were prepared in concentrations of 0.0 to 3.0 μmoles of base/ 2.0 ml. of 2 N perchloric acid. Each sample was placed on a cation exchange column and eluted with 2 N HCl (See text). 5.0 ml. fractions of the eluent were collected and the optical density of each recorded and plotted against the ml. of eluent. The area under the resultant peaks was calculated and appears here plotted against the amount of base in each sample.
o - Adenine; x - Guanine; ⊖ - Uracil; ● - Cytosine and Cytosine nucleosides.

Ultra-violet absorbing materials in the fractions were determined and recorded as in the case of the standard solutions. A typical chromatogram is presented in Fig. 10.

Isotopic Experiments

A study of the incorporation of various guanine derivatives into nuclear RNA was initiated for the purpose of determining which compound could be utilized most effectively in an extensive study of the rate of incorporation of RNA precursors into nuclear RNA. A group of 18 or 19 female Sprague-Dawley albino rats were wounded and maintained in the usual manner. On the day of sacrifice, 8 days after wounding, each animal was given an injection i.p. of 5 microcuries of the labeled compound (guanylic acid-8-C^{14}, guanosine-8-C^{14}, or guanine-8-C^{14}) and sacrificed 3 hours later. The wound was excised as quickly as possible and frozen in liquid nitrogen; from this point on, the separation of RNA was the same as for non-isotopic experiments. Aliquots of the RNA fraction isolated were placed in planchets, neutralized, and evaporated to dryness; these were then counted in a thin window gas flow assembly with automatic sample changer. Samples were corrected for background, dead time, and instrument efficiency.

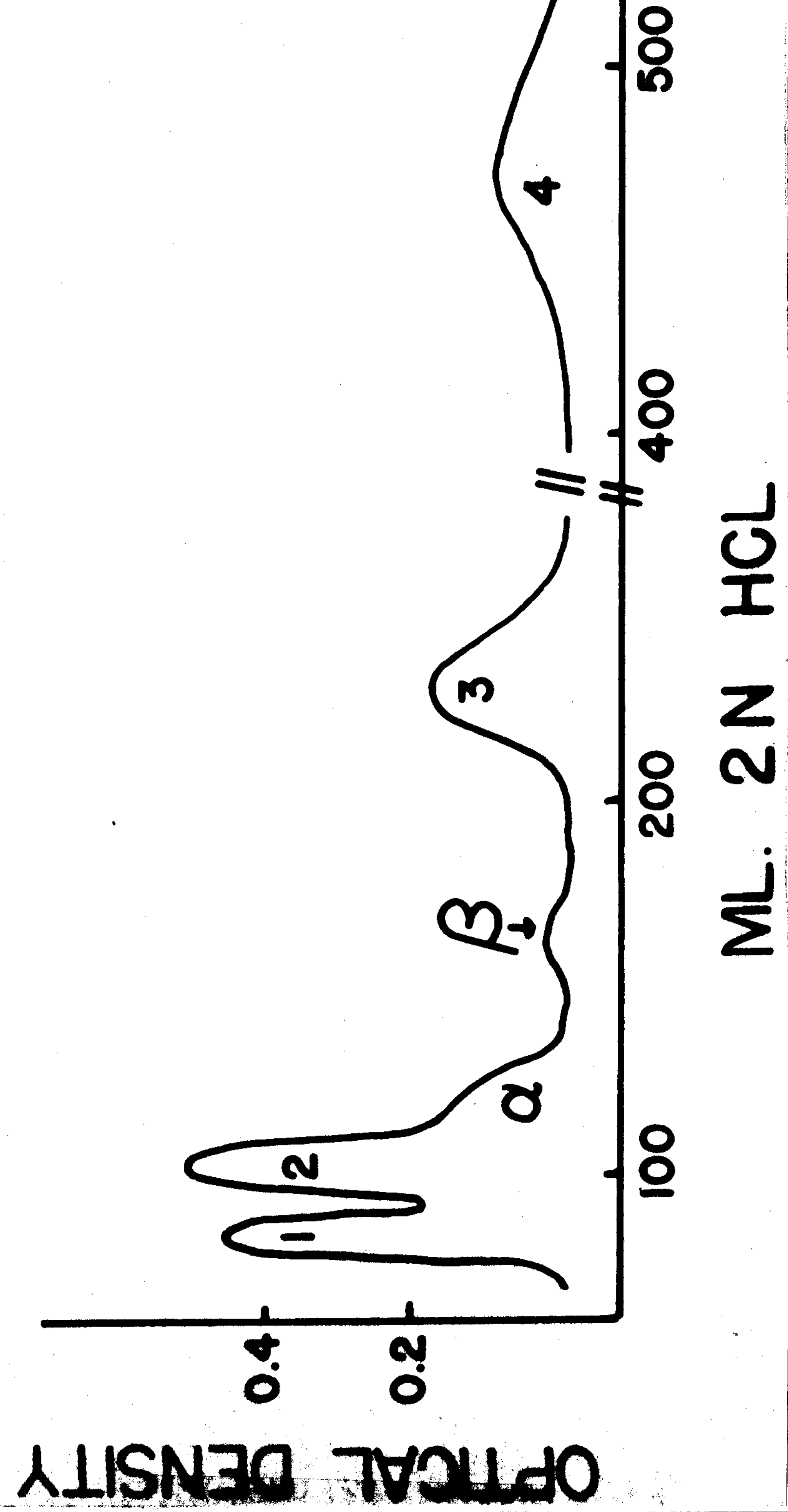


Fig. 10: Chromatogram obtained by plotting optical density against ml. eluent. A 2.0 ml. sample of nuclear RNA isolated from a pooled sample of tissue collected 5 days after wounding was placed on the column and eluted as described on p. 43. Peak no. 1 represents uracil and any uridine nucleosides which may be present. Peak no. 2 represents cytosine; the portion of this peak is thought to represent cytidine. The peak is believed to represent a methyl cytosine. Peak no. 3 represents guanine and peak no. 4 represents adenine.

CHAPTER V

RESULTS AND DISCUSSION

Interpretation of the data presented can best be made after considering some of the limitations of the methods and techniques employed in obtaining the nuclear fraction from regenerating wound tissue.

In general, techniques developed for the isolation of nuclear material are dependent on a difference in specific gravity between the nuclei and the cytoplasmic components of the cell. Much of the experimental work on nuclear material has been done with calf thymus nuclei, because the specific gravity of the nucleus is appreciably different from that of the cytoplasm. In this tissue, the nucleus comprises 60% of the cell; hence, separation of the nuclear material can be achieved rather easily and in good yield. In granulation tissue, however, the specific gravity of the nucleus is thought to be closer to that of the cytoplasm. Also, the nucleus comprises a smaller portion of the total cellular material. These properties make the isolation of nuclear material from granulation tissue much more difficult.

The isolation of nuclear material from granulation tissue presents additional problems because of the constantly changing character of the tissue. Five days after wounding, the tissue appears to have a high water content and low fiber and cell content as compared to the same tissue three days later, eight days after wounding. Five days after wounding, the tissue is very thin and difficult to disect from the animal;

eight days after wounding, the tissue is much firmer and may easily be disected from the wound area. At the latter time, the water content appears to have decreased and the cell and fiber content appears to have increased. By the twelfth day after wounding, the wound has contracted appreciably and lost even more water, while the collagen fiber content has increased appreciably. Essentially, therefore, one must work with three different types of tissue, each characteristic of a certain stage of the regenerative process.

Homogenization of the tissue in such a way as to liberate intact nuclei is another difficulty encountered in the isolation of nuclear material. It was observed that the use of a glass homogenizer resulted in a negligible yield of intact nuclei. Use of a Virtis homogenizer resulted in contamination of the nuclear material with whole cells and appreciable amounts of large collagen fragments. It was finally decided to freeze the tissue in liquid nitrogen, then crush it in the frozen state with a mortar and pestle. This technique permitted fragmentation of the collagen fibers while leaving the nuclei largely intact.

Centrifugation of suspensions of crushed regenerating wound tissue from organic solvents of varying specific gravity was attempted as a method of isolating the nuclear material. Because of the differences in the properties of the regenerating wound tissue at different stages of formation, it was found that a new centrifugation technique would have to be developed for each type of tissue collected during the regenerative process. Also, the losses during execution of this technique were so great as to necessitate the use of inconveniently large numbers

of animals to obtain reasonable yields of nuclear material (6).

The use of aqueous solutions for the suspension of tissue homogenates e.g. citric acid buffers or sodium chloride solutions, has been observed to extract some water soluble components from the nuclei. These techniques were avoided, as some extraction of RNA from the nuclear material during the isolation procedure was to be expected (19, 20).

Isolation of nuclei by centrifugation from dilute sucrose solutions has been used successfully (20, 42, 76). In some tissues, this technique has permitted the isolation of intact nuclei with very little contamination by cytoplasmic material and little loss of nuclear components. Isotonic solutions (0.25 M) have been utilized most frequently, since the nuclei remain intact in such solutions. Hypertonic solutions (20) have been utilized in some cases in an attempt to concentrate the nuclear material and increase its specific gravity. This procedure, when utilized with granulation tissue, resulted in poor separations due to imbedding of the shrunken nuclei in collagen fragments. Addition of Ca^{++} ions to the sucrose solutions has been shown to reduce agglutination of the nuclei with cytoplasmic debris, thereby facilitating the isolation of a relatively cytoplasm-free nuclear fraction.

After a rather thorough investigation of the available techniques, it was decided that isolation of nuclear material from 0.25 M sucrose solution containing 0.003 M Ca^{++} would be best suited to this series of experiments. This procedure permitted the nuclei to be maintained at their maximal intact size while effecting at least gross separation of collagen fragments, nuclei, and lighter cytoplasmic components.

Further purification of nuclei presented additional problems. A combined aqueous-nonaqueous technique involving isolation of the nuclear material in 0.25 M sucrose containing 0.003 M Ca^{++}, lyophilization, and further purification in organic solvents did not yield either intact nuclei or appreciable purification, as observed by histological examination. Resuspension of the crude nuclear fraction in 0.44 M sucrose resulted in rupturing of the nuclei. Layering techniques were also attempted. These consisted of layering a portion of the homogenate (0.25 M sucrose as the suspending medium) over a sucrose solution of higher specific gravity. Presumably, light cytoplasmic debris would be maintained at the interface or by the more dense solution, while the heavier nuclei would sediment to the bottom of the more dense solution upon careful centrifugation. This procedure must be repeated several times to be effective; however, the repeated suspension and centrifugation of the nuclei resulted in either agglutination or rupture of the nuclei.

It was finally decided that a crude unpurified nuclear fraction would be utilized in this series of experiments. (See Chapter IV for rationale behind this decision.)

Values obtained by measurement of purine-bound ribose, purine-bound deoxyribose, and total nuclear protein present in the nuclear fraction of regenerating wound tissue are summarized in Tables III, IV, and V. The variable conditions for each experiment and the raw values obtained, as well as calculations of RNA and DNA on the basis of nuclear protein content, are presented.

TABLE III

D-RIBOSE, D-2-DEOXYRIBOSE, AND TOTAL PROTEIN IN NUCLEAR NUCLEIC ACIDS 5 DAYS AFTER WOUNDING

No. Rats	μmoles D-Ribose	μmoles D-2-Deoxy-ribose	mg. Protein	μ moles RNA Purine Nucleotides / mg. Protein	μmoles DNA Purine Nucleotides / mg. Protein	RNA / DNA
20	7.34	8.9	109	0.067	0.082	0.83
5	0.88	3.2	14	0.063	0.23	0.28
5	3.83	13.0	75	0.051	0.17	0.30

TABLE IV

D-RIBOSE, D-2-DEOXYRIBOSE, AND TOTAL PROTEIN IN NUCLEAR NUCLEIC ACIDS 8 DAYS AFTER WOUNDING

No. Rats	μ moles D-Ribose	μ moles D-2-Deoxy-ribose	mg. Protein	μ moles RNA Purine Nucleotides / mg. Protein	μ moles DNA Purine Nucleotides / mg. Protein	RNA / DNA
19*	14.52	3.8	25	0.592	0.13	3.82
18*	12.00	---	98	0.122	----	----
18*	12.07	49.0	280	0.043	0.18	0.247
22	12.20	28.6	333	0.037	0.09	0.429
10	3.42	8.3	50	0.068	0.17	0.412
10	1.58	3.8	18	0.088	0.21	0.415
10	5.42	10.7	74	0.073	0.15	0.506
9	9.92	12.5	107	0.093	0.12	0.793
9	6.13	10.9	63	0.097	0.17	0.562

* Isotopic Experiments

TABLE V

D-RIBOSE, D-2-DEOXYRIBOSE, AND TOTAL PROTEIN IN NUCLEAR NUCLEIC ACIDS 12 DAYS AFTER WOUNDING

No. Rats	μ moles D-Ribose	μ moles D-2-Deoxy-ribose	mg. Protein	μ moles RNA Purine Nucleotides / mg. Protein	μ moles DNA Purine Nucleotides / mg. Protein	RNA / DNA
27	4.67	6.4	58	0.081	0.11	0.73
18	5.42	12.0	54	0.103	0.22	0.45

The values obtained by measurement of the purine-bound ribose have been used as a measure of the total RNA content of the nuclear fraction. Since only a portion of the RNA is measured by this reaction, the actual RNA content in the nuclear fraction is undoubtedly appreciably higher. However, the objective of these experiments is not primarily concerned with the absolute amount of RNA present at a given time, but rather with the change in RNA content over a period of time. Any changes occurring in nuclear RNA content will be proportional to the change in purine-bound ribose. For convenience, then, the values obtained in the determination of purine-bound ribose will be looked upon as a measure of total nuclear RNA. Similarly, the determination of purine-bound deoxyribose by means of the indole reaction (12) will be used as a measure of total DNA.

There appears to be considerable variation in the raw values as determined by measurement of ribose and deoxyribose, even when similar amounts of tissue are used i.e. when pooled samples consisting of tissues from equal numbers of rats are used. When these data are calculated on a protein basis, however, they are in much better agreement.

The ratio of μ moles of RNA purines/mg. nuclear protein, μ moles DNA purines/mg. nuclear protein, and RNA/DNA have been obtained by statistical treatment of the raw data and are presented in Table VI. Micromoles of RNA (purines + pyrimidines)/mg. nuclear protein, as calculated from the purine/pyrimidine ratios in Table IX, is also included in Table VI. The change in ratio of RNA/DNA with time during the regenerative process is depicted graphically in Figure 11.

TABLE VI

NUCLEIC ACIDS IN THE NUCLEI OF CELLS IN REGENERATING WOUND TISSUE

	5 Days after Wounding	8 Days after Wounding	12 Days after Wounding
$\frac{\mu \text{ moles RNA purine}}{\text{mg. Nuclear Protein}}$	0.065 ± 0.00	0.071 ± 0.03	0.090 ± 0.01
$\frac{\mu \text{ moles DNA purine}}{\text{mg. Nuclear Protein}}$	0.161 ± 0.06	0.165 ± 0.01	0.166 ± 0.08
$\frac{\mu \text{ moles RNA purine}}{\mu \text{ moles DNA purine}}$	0.284 ± 0.01	0.465 ± 0.07	0.590 ± 0.19
* $\frac{\mu \text{ moles RNA purines pyrimidines}}{\text{mg. Nuclear Protein}}$	0.134	0.162	0.223

* Derived value, using pu/py ratios in Table VIII.

Fig. 11: Change in Nuclear RNA/DNA Ratio of Regenerating Wound Tissue with Time after Wounding.

The DNA content of the nuclear fraction of regenerating wound tissue can be seen to remain essentially constant over the 12 day period of observation. This is in contrast to the findings of Williamson and Guschlbauer on the DNA content of whole tissue homogenates of regenerating wound tissue (92). This apparent discrepancy can be resolved by considering that in each situation, the DNA values are calculated on the basis of total protein or tissue nitrogen. In the nuclear fraction, the amount of total protein present is of the same order of magnitude throughout the period of observation. However, in the whole tissue homogenate, the amount of tissue nitrogen or total protein increases as tissue regeneration progresses. As a result, the ratio of DNA/tissue nitrogen increases in the early stages of wound healing when cellular proliferation is intense and the rate of DNA formation is high; it then reaches a maximum and declines when the DNA content of the tissue has reached a constant level and deposition of extracellular protein continues to increase.

The RNA content on the basis of protein in the nucleus can be seen to increase very slightly but steadily throughout the twelfth day after wounding. This parallels the findings of Guschlbauer and Williamson (92) on whole tissue homogenates very closely, except that the total amounts of RNA in the nuclear fraction are much smaller, as would be expected. A similar increase in RNA content was observed by Hosoda (44) in whole tissue homogenates from mice. It appears that the changes observed in nuclear RNA levels contribute only a small fraction of the total change observed in the RNA content of cells during the regenerative process.

The amount of nuclear RNA as compared to DNA can be seen to increase almost linearly with the course of tissue regeneration. These findings would seem to indicate that an appreciable amount of protein synthesis, with consequent demand for nuclear RNA, occurs throughout the wound healing period. This protein-synthesizing activity most likely represents both collagen synthesis and turnover of nuclear proteins. At this point, it cannot be determined which process may be the more significant.

Results of Chromatography of Nitrogenous Bases

The chromatogram showing the resolution of the nitrogenous base components of nuclear RNA isolated from wound tissue five days after wounding is represented in Figure 10 (p. 46). The numbered peaks represent uracil, cytosine, guanine, and adenine respectively. In calibrating the column, it was observed that cytidine was eluted in the same position as the α component of the cytosine peak. This seems to indicate incomplete hydrolysis of pyrimidine nucleotides under the given conditions. Uridine, uracil, thymine, and thymidine all have no charge and migrate together; therefore, it is not possible to observe whether the RNA fraction is contaminated with DNA (the only potential source of thymine or thymine nucleosides). The β peak has not been investigated, but is thought to represent a methyl cytosine.

The amounts of bases obtained from nuclear RNA at various periods during the regenerative process are shown in Table VII. There appears to be a slight preponderance of pyrimidines early in the regenerative process which increases in the later stages. This is in contradistinction

TABLE VII

BASE COMPOSITION OF NUCLEAR RNA IN REGENERATING WOUND TISSUE

Base	Days after Wounding					
	5 Days		8 Days		12 Days	
	μmoles	%	μmoles	%	μmoles	%
Uracil	4.4	15.4	19.5	29.5	2.2	17.6
Cytosine Cytidine	10.3	36.2	17.7	26.6	5.3	42.4
Guanine	7.3	25.6	17.3	26.0	3.2	25.6
Adenine	6.5	22.8	12.0	18.0	1.8	14.4

to the observations of Williamson and Guschlbauer (94), as can be seen by the comparison of purine/pyrimidine base ratios in Table VIII. In the data of Williamson and Guschlbauer, it appears that the purine/pyrimidine ratio is essentially unity during the early stages of wound healing. By the twelfth day, both sets of values compare favorably with each other.

In Table IX, the amino acid composition of rat skin collagen is presented according to the findings of Piez (66). One of the possible code triplets that would be expected to be found in messenger RNA concerned with collagen biosynthesis is included for each amino acid. The relative amounts of purines and pyrimidines (on a percentage basis) have been calculated and are shown in succeeding columns. The purine/pyrimidine ratio for rat skin collagen has been calculated from these data to be approximately 0.82. This would seem to indicate that maximum collagen precursor formation takes place between the fifth and the eighth days after wounding. The use of other possible triplet codes for the various amino acids present in collagen may yield a calculated purine/pyrimidine ratio in closer agreement with the findings of Williamson et.al. (94). It has been observed that the triplet codes for the basic amino acids lysine and arginine, as well as that for histidine, contain a purine-pyrimidine ratio of greater than one. It appears that nuclear RNA containing high amounts of purines would be primarily concerned with nuclear protein synthesis, while that having a somewhat high concentration of pyrimidines would be primarily concerned with collagen formation.

The ratios obtained by Williamson and Guschlbauer (94) may show a somewhat retarded increase in pyrimidine content due to the fact that the

TABLE VIII

RATIO OF PURINES TO PYRIMIDINES

IN RNA OF REGENERATING WOUND TISSUE

Days after Wounding	Purine-Pyrimidine Ratio	
	Current* Expts.	Published** Data
5 Days	0.94	1.11
8 Days	0.78	1.05
12 Days	0.67	0.70

* Nuclear RNA

** Total Cellular RNA (94)

TABLE IX

PERCENT OF NITROGENOUS BASES IN CODE TRIPLETS REQUIRED FOR COLLAGEN SYNTHESIS (50, 66)

Amino Acid	% Of Collagen	Code Triplet	% Pu	% Py
Proline				
Hydroxyproline	22.2	CCU	---	22.2
Aspartic acid	4.6	GUA	3.0	1.6
Threonine	1.96	CAC	0.6	1.2
Serine	4.3	UCU	---	4.3
Glutamic acid	7.1	AGU	4.8	2.4
Glycine	33.1	UGG	22.1	11.0
Alanine	10.6	CCG	3.5	7.1
Valine	2.4	UGU	0.8	1.6
Methionine	0.8	UGA	1.6	0.8
Isoleucine	10.8	UAU	3.6	7.2
Leucine	2.4	UUG	0.8	1.6
Tyrosine	0.2	AUU	0.1	0.2
Phenylalanine	1.1	UUU	---	1.1
Lysine				
Hydroxylysine	3.4	AAA	3.4	---
Histidine	0.5	ACC	0.2	0.3
Arginine	5.1	CGC	1.7	3.4

animals used in that series of experiments were maintained on a protein-free diet; hence, all metabolic processes would be retarded and collagen biosynthesis would be appreciably delayed.

Results of Isotopic Studies

The data obtained from the isotopic studies made with guanylic acid-8-C^{14}, guanosine-8-C^{14}, and guanine-8-C^{14} are summarized in Table X. The specific activity (c/m/μmole RNA) of the nuclear RNA isolated after administration of guanosine-8-C^{14} can be seen to be more than ten times as much as that of nuclear RNA isolated after administration of guanylic acid-8-C^{14}. Essentially no incorporation of guanine-8-C^{14} into nuclear RNA was observed over the three hour period.

Determination of the specific activity of the DNA isolated after administration of guanylic acid-8-C^{14} and of guanine-8-C^{14} (c/m/μmole DNA) revealed some incorporation into the DNA fraction. Without further experimentation, it is difficult to judge whether these observations are of significance, or whether they are the result of poor experimental technique. There is reason to suspect the latter in the case of values obtained after administration of guanylic acid-8-C^{14}, as the amount of DNA isolated in that experiment was much lower than the amounts isolated in other similar experiments (See Table IV). The amount of DNA isolated after administration of guanine-8-C^{14} appears to be somewhat higher than the values obtained in other experiments (See Table IV). The specific activity of the DNA isolated after administration of guanine-8-C^{14} is very low; however, when it is considered that no incorporation into RNA

TABLE X

INCORPORATION OF LABELED PURPORTED RNA PRECURSORS INTO NUCLEAR RNA OF REGENERATING WOUND TISSUE

C^{14}-Guanine Derivative	Total c/m RNA	Total μmoles RNA	c/m per μmole RNA	mg. Protein	c/m per mg. Protein
Guanylic Acid-8-C^{14}	250	14.52	17	24.5	10
Guanosine-8-C^{14}	3500	12.00	292	98	35.7
Guanine-8-C^{14}	0	12.07	0	280	0

Conditions: 18 rats each received 5 microcuries of labeled compound 8 days after wounding; all samples collected 3 hours after injection.

could be detected, even such a low level of incorporation into the DNA fraction may be significant. Further experimentation using well developed techniques is necessary before any valid conclusions as to the incorporation of C^{14}-guanine derivatives into DNA can be made.

Williamson and Guschlbauer (94) have shown in measurements of total cellular RNA that the ratio of purines/pyrimidines is approximately equal to unity during the early stages of wound healing. If this ratio is maintained in nuclear RNA, it would seem that the specific activity of guanosine-8-C^{14} is of the order of 150 c/m/μmole total RNA. The specific activity of P^{32} under the same conditions is approximately 1200 c/m/μmole total RNA. Several reasons may be considered for this discrepancy:

1. The discrepancy may be due merely to the different dose levels of isotope administered e.g. 80 microcuries of P^{32} per animal and 5 microcuries of guanosine-8-C^{14} per animal. It is difficult to estimate the percent of the total dose which is incorporated into the nuclear RNA fraction, particularly in the case of P^{32}, since so many other types of compounds are known to utilize it at the same time.

2. The difference in amount of incorporation observed may be indicative of a difference in the rate of incorporation of the two compounds without necessarily implying a change in the turnover rate of nuclear RNA. It is possible that the exchange of P^{32} and RNA-P^{31} may be a partially non-enzymic reaction, particularly at the dosage administered; whereas the incorporation of guanosine-8-C^{14} is dependent upon two enzymic reactions, namely, phosphorylation of guanosine to the nucleoside triphosphate

and incorporation of the latter into nuclear RNA by the action of RNA polymerase. Hence, in a given time, the incorporation of guanosine-8-C^{14} may appear to be slower because of the number and rate of the reactions involved in its incorporation.

McEwen, et.al. (58), in studies of the incorporation of adenine derivatives into calf thymus nuclei *in vitro*, have reported several observations in agreement with those presented here. When such nuclei were incubated with C^{14}-AMP or C^{14}-ADP at 37° C. for periods of one hour or less, the label was isolated primarily in the nucleoside fraction. Very little labeled ATP or RNA was isolated. Addition of an excess of C^{12}-adenosine to the same mixture resulted in a 60% reduction of incorporation of C^{14}.

It appears that similar conditions exist *in vivo* in the nuclei of regenerating wound tissue cells. Guanosine-8-C^{14} is incorporated to a much greater extent than either its monophosphorylated derivative or guanine itself. However, unlike McEwen's findings, an appreciable amount of the label appears to be incorporated from the intranuclear nucleoside pool into nuclear RNA. This may have been due in part to a longer incubation period (three hours), or to some factor present under *in vivo* conditions, but not present under *in vitro* conditions. No determination of intranuclear levels of free nucleosides or nucleotides was made, so it is difficult to estimate what percentage of the dose incorporated into the nucleus was further incorporated into nuclear RNA.

McEwen postulated that nucleotides must be hydrolyzed to nucleosides before entry into the nucleus can take place; within the nucleus, the

nucleosides must then be rephosphorylated in order to be incorporated into nuclear RNA. Incorporation of AMP-C^{14} into the nucleus was observed under *in vitro* conditions and incorporation of GMP-C^{14} into the nucleus was observed under *in vivo* conditions. The rate of incorporation in both cases was slower than for the analogous nucleoside derivatives, thereby giving evidence for the mechanism postulated above.

Adenine-C^{14} was observed to be incorporated under *in vitro* conditions, while guanine-C^{14} was not incorporated under *in vivo* conditions. Several reactions are known to involve the conversion of adenine to adenine nucleotides in mammals (95); it may be that analogous enzymes for guanine are not present in the cells of regenerating wound tissue, thereby accounting for the negligible guanine incorporation observed in these experiments.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Regenerating wound tissue obtained from female rats was homogenized and the nuclear material isolated. The RNA of the nucleus was separated from the DNA by a modification of the method of Schmidt and Thannhauser (74). Total RNA in the preparations was measured by means of the orcinol reaction for purine-bound ribose (11), while the DNA was determined by the indole reaction for purine-bound deoxyribose (12). The isolated RNA was hydrolyzed; the nitrogenous bases and nucleosides arising from this procedure were separated by ion-exchange chromatography on Dowex resins (15). Purine/pyrimidine base ratios were then calculated.

In other experiments, guanylic acid-8-C^{14} or guanosine-8-C^{14} or guanine-8-C^{14} was administered *in vivo* three hours previous to sacrificing each animal. The isolation of nuclear RNA was carried out exactly as in the earlier experiments. In addition to the determinations described, aliquots of the isolated RNA fraction were neutralized and the activity measured.

The observations made in this series of experiments may be summarized as follows:

1. On the basis of nitrogen content, the DNA content of the nuclear fraction of regenerating wound tissue remained essentially constant throughout the period of tissue regeneration studied, while a slight increase in the RNA content of the same fraction was observed.

2. Acidic hydrolysis of the isolated RNA resulted in the liberation of free purine bases and a mixture of free pyrimidine bases and pyrimidine nucleosides, as observed by ion-exchange chromatography. Trace amounts of a substance suspected to be a methyl cytosine were also isolated during the chromatographic separations.

3. Purine/pyrimidine base ratios, calculated from the chromatographic data, were compared with the value expected for the hypothetical messenger-RNA required for collagen synthesis. This comparison appeared to indicate that nuclear RNA may be primarily concerned with nuclear protein synthesis during the early stages of the wound tissue regeneration and with collagen biosynthesis during the later stages.

4. The purine/pyrimidine ratios calculated from the data in this series of experiments are somewhat lower than those previously published (94) during the early stages of wound healing; however, the values are in good agreement in the later stages of regeneration. This difference may be due to an artifact which results when data obtained by various methods are compared, or it may be due to the fact that the animals used in this series of experiments were maintained on a normal diet, while those used in earlier experiments (94) were maintained on a protein-free diet.

5. Guanosine-8-C^{14} is incorporated to a much greater extent into nuclear RNA of wound tissue than either guanine-8-C^{14} or guanylic acid-8-C^{14}.

APPENDIX

mg. ---------------- milligram

ml. ---------------- milliliter

μ ---------------- micro-

M ---------------- molar solution

N ---------------- normal solution

Pu ---------------- purine

Py ---------------- pyrimidine

A ---------------- adenine, or derivative thereof.

G ---------------- guanine, " "

C ---------------- cytosine, " "

U ---------------- uracil, " "

BIBLIOGRAPHY

1) Allen, E. H., Glassman, E., Cordes, E., and Schweet, R. S., J. Biol. Chem., 235, 1061 (1960).

2) Allen, E. H., Glassman, E., and Schweet, R. S., J. Biol. Chem., 235, 1068 (1960).

3) Allfrey, V. G., and Mirsky, A. C., Proc. Natl. Acad. Sci., 43, 821 (1957).

4) Allfrey, V. G., and Mirsky, A. E., Proc. Natl. Acad. Sci., 45, 1325 (1959).

5) Barakina, N. F., Doklady Akad. Nauk., 86, 1053 (1952).

6) Behrens, M., Z. Physiol. Chem., 209, 59 (1932).

7) Bessman, M. J., Lehman, I. R., Simms, E. S., and Kornberg, A., J. Biol. Chem., 233, 171 (1958).

8) Blumfeld and Gallop, P. M., Fed. Proc., 21, 409 (1962).

9) Boedtker, H., and Doty, P., J. Am. Chem. Soc., 78, 4267 (1956).

10) Bradbury, S., and Meek, G. A., Quart. J. Microscop. Sci., 99, 143 (1958).

11) Ceriotti, G., J. Biol. Chem., 198, 297 (1952).

12) Ceriotti, G., J. Biol. Chem., 214, 59 (1955).

13) Chantrenne, H., in "The Biosynthesis of Proteins", Pergamon Press, New York, p. 57, 1961.

14) Chapman, J. A., J. Biophys. Biochem. Cytol., 9, 639 (1961).

15) Cohn, W. E., Sci., 109, 377 (1949).

16) David, E. W., Koningsbefger, W. W., and Lipmann, F., Arch. Biochem. Biophys., 65, 21 (1956).

17) De Moss, J. A., Genuth, S. M., and Novelli, G. D., Proc. Natl. Acad. Sci., 43, 325 (1956).

18) Dounce, A. L., J. Biol. Chem., 151, 235 (1943).

19) Dounce, A. L., J. Cellular. Comp. Physiol., 39, Suppl. 2, 43 (1952).

20) Dounce, A. L., in "The Nucleic Acids", (Chargaff and Davidson, Eds.), p. 94-120, Academic Press, N. Y., 1955.

21) Faure-Fremiet, E., C. R. Soc. Biol., Paris, 113, 715 (1933).

22) Folin, O., and Ciocalteu, V., J. Biol. Chem., 73, 627 (1927).

23) Gallop, P. M., Seifter, S., and Meilman, E., Nature, 183, 1659 (1959).

24) Gallop, P. M., and Seifter, S., in "Technical Seminar on Collagen" (N. Ramanathan, Ed.), Wiley (Interscience), New York, in press.

25) Goldthwait, D. A., Biochem. Biophys. Acta, 30, 643 (1958).

26) Grassman, W., Hannig, K., and Schleyer, M., Z. Physiol. Chem. Hoppe-Seyler's, 322, 71 (1960).

27) Green, N. M., and Lowther, D. A., Biochem. J., 71 55 (1959).

28) Grobbelaar, N., Pollard, J. K., and Steward, F. C., Nature, Lond., 175, 703 (1955).

29) Gross, J., in "Comparative Biochemistry", (M. Florkin and H. S. Mason, Eds.), Academic Press, New York, 1963.

30) Gross, J., Fed. Proc., 17 62 (1958).

31) Gross, J., Highberger, J. H., and Schmitt, F. O., Proc. Natl. Acad. Sci., Wash., 41, 1 (1955).

32) Gross, J., J. Biophys. Biochem. Cytol., 2, 26. (1956).

33) Gross, J., J. Exp. Med., 107, 265 (1958).

34) Guschlbauer, W., Ph.D. Dissertation, Loyola Univ., p. 16-31, 1961.

35) Guschlbauer, W., and Williamson, M. B., Canad. J. Biochem. Physiol., 41, 820 (1963).

36) Harkness, R. D., Biological Reviews, 36, 399 (1961).

37) Hecht, L. I., Stephenson, M. L., and Zamecnik, P. C., Biochem. Biophys. Acta, 29, 460 (1958).

38) Hecht, L. I., Stephenson, M. L., and Zamecnik, P. C., Proc. Natl. Acad. Sci., 45, 505 (1959).

39) Hoagland, M. B., Biochim. Biophys. Acta, 16, 288 (1955).

40) Hoagland, M. B., Keller, E. B., and Zamecnik, P. C., J. Biol. Chem.

41) Hoagland, M. B., Zamecnik, P. C., and Stephenson, M. L., Biochim. Biophys. Acta, 24, 215 (1957).

42) Hogeboom, G. H., Schneider, W. C., and Striebach, M. S., J. Biol. Chem., 196, 111 (1952).

43) Hopkins, J., Proc. Natl. Acad. Sci., 45, 1461 (1959).

44) Hosoda, Y., Keio J. Med., 9, 261 (1960).

45) Hutchison, W. C., and Munro, H. N., The Analyst, 86, 768 (1961).

46) Jackson, D. S., Leach, A. A., and Jacobs, S., Biochim. Biophys. Acta, 27, 418 (1958).

47) Jackson, S. F., in "Bone as a Tissue" (K. Rodahl, J. T. Nickelson, and E. M. Brown, Eds.), p. 165, McGraw-Hill, New York, 1960.

48) Jackson, S. F., Proc. Roy. Soc. B., 144, 556 (1956).

49) Jacob, F., and Monod, J., J. Mol. Biol., 3, 318 (1961).

50) Jukes, T. H., Am. Sci., 51, 227 (1963).

51) Karrer, H. E., J. Ultrastructure Res., 2, 96 (1958).

52) Keller, E. B., and Zamecnik, P. C., J. Biol. Chem., 221, 45 (1956).

53) Kornberg, A., Sci., 131, 1503 (1960).

54) Kurland, C. G., J. Mol. Biol., 2, 23 (1960).

55) Lengyel, P., Speyer, J. F., and Ochoa, S., Proc. Natl. Acad. Sci., 47, 1936 (1961).

56) Lipmann, F., Hulsmann, W. C., Hartmann, G., Boman, H. G., and Acs, G., J. Cell. Comp. Physiol., Suppl. 1, 75 (1959).

57) Logan, R., Ficq, A., and Errera, M., Biochim. Biophys. Acta, 31, 402 (1959).

58) Maas, W. K., and Novelli, G. D., Arch. Biochem. Biophys., 43, 236 (1953).

59) McEwen, B. S., Allfrey, V. G., and Mirsky, A. E., J. Biol. Chem., 238, 758 (1963).

60) Mirsky, A. E., Osawa, S., and Allfrey, V. G., Cold Spring Harbor Symp., 21, 49 (1956).

61) Nirenberg, M. W., and Matthaei, J. H., Proc. Natl. Acad. Sci., 47, 1588 (1961).

62) Nirenberg, M. W., Scientific Am., 208, 80 (1963).

63) Ochoa, S., in "Horizons in Biochemistry", (M. Kasha and B. Pullman, Eds.), Academic Press, New York, p. 160, (1962).

64) Orekovitch, B. V., Proc. 2nd. Int. Congr. Biochem., 106 (1952).

65) Palladina, L. I., and Gudina, A. M., Ukrain. Biokhim. Zhur., 28, 442 (1956).

66) Piez, L. A., Eigner, E. A., and Lewis, M. S., Biochemistry, 2, 58 (1963).

67) Porter, K. R., and Pappas, J. D., J. Biophys. Biochem. Cytol., 5, 153 (1959).

68) Raekallio, J., Ann. Med. Exptl. Biol. Fennial (Helsinki), 30, Suppl. 6, (1961).

69) Rapport, D., Canzanelli, A., and Guild, R., Am. J. Physiol., 162, 421 (1950).

70) Rendi, I., Exptl. Cell. Res., 19, 480 (1960).

71) Rich, A., and Crick, F. H. C., J. Mol. Biol., 3, 483 (1961).

72) Risebrough, R. W., Tissieres, A., and Watson, J. D., Proc. Natl. Acad. Sci. U. S., 48, 430 (1962).

73) Rudas, B., Acta Physiol. Acad. Sci. Hungaricae, 8, 253 (1955).

74) Schmidt, G., and Thannhauser, S. J., J. Biol. Chem., 161, 83 (1945).

75) Schmitt, F. O., Gross, J., and Hignberger, H., Proc. Natl. Acad. Sci. Wash., 39, 459 (1953).

76) Schneider, R. M., and Peterman, M. L., Cancer Res., 10, 751 (1950).

77) Singer, M. F., and Cantoni, G. L., Biochim. Biophys. Acta, 39, 182 (1960).

78) Spirin, A. S., in "Progress in Nucleic Acid Research", (J. N. Davidson and Waldo H. Cohn, Eds.), Academic Press, New York, p. 301, 1963.

79) Stetten, M. R., J. Biol. Chem., 181, 31 (1949).

80) Tissieres, A., Watson, J. D., Schlessinger, D. A., and Hollingworth, B. R., J. Mol. Biol., 1, 221 (1959).

81) Tsanev, R., Doklady Bolg. Akad. Nauk., 11, 53 (1958).

82) Tsanev, R., Invest. 1st Biol. M. Papoff, Bolg. Nauk. Acad., 7, 193 (1956).

83) Tsanev, R., Bulg. Akad. Sci. Zhur., 455 (1958).

84) Tsanev, R., and Vulchanov, V. H., Doklady Bolg. Akad. Nauk., 8, 61)1955).

85) Volkin, E., Astrachan, L., and Countryman, J. L., Virology, 6, 545 (1958).

86) Warner, R. C., J. Biol. Chem., 236. PC 43. (1957).

87) Wasserman, F., Ergebn. Anat. Entwgesch., 35, 240 (1956).

88) Watson, J. D., and Crick, F. H. C., Nature, 171, 545 (1953).

89) Weiss, P., and Ferris, W., Exp. Cell Res., 6, 546 (1954).

90) Weiss, P., and Ferris, W., Proc. Natl. Acad. Sci., 40, 528 (1954).

91) Williamson, M. B., and Fromm, H. J., J. Biol. Chem., 212, 705 (1955).

92) Williamson, M. B., and Guschlbauer, W., Arch. Biochem. Biophys., 100, 251 (1962).

93) Williamson, M. B., and Guschlbauer, W., J. Biol. Chem., 236, 1463 (1961).

94) Williamson, M. B., and Guschlbauer, W., Nature, 192, 454 (1961).

95) West, E. S., and Todd, W. R., in "Textbook of Biochemistry", 3rd. ed., Macmillan Co., New York, p. 1145, 1961.

APPROVAL SHEET

The thesis submitted by Eleanore M. Hertel has been read and approved by three members of the faculty of the Graduate School.

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

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

Jan 14, 1964
Date

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