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**Nucleic Acids in Wound Healing**

Wilhelm Guschlbauer

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NUCLEIC ACIDS IN WOUND HEALING

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

June 1961

LOYOLA UNIVERSITY MEDICAL CENTER
TO

MY PARENTS
Resignation in Bezug auf das Erkennen der Welt ist für mich nicht der rettungslose Fall in einen Skeptizismus, der uns wie ein steuerloses Wrack in dem Leben dahintreiben lässt. Ich sehe darin die Wahrhaftigkeitsleistung, die wir wagen müssen, um von da aus zu der wertvollen Weltanschauung, die uns vorschwebt, zu gelangen. Alle Weltanschauung, die nicht von der Resignation des Erkennens ausgeht, ist gekünstelt und erdichtet, denn sie beruht auf einer unzulässigen Dautung der Welt.

Albert Schweitzer

Vorrede zu "Kultur und Ethik"
(Kulturphilosophie II)
Wilhelm Guschlbauer was born in Paris, France, October 8, 1932. He graduated from "Akademisches Gymnasium" in Vienna, Austria, in June 1960.

He completed his undergraduate requirements at the University of Vienna, Austria, with the Doctorandum. In 1955, he was appointed Research and Teaching Assistant in the Department of Microbiology and Biochemical Technology at the Technische Hochschule in Vienna. He served as a Consulting Associate to the Austrian Sugar Corp. during 1957 - 1958.

In 1958, he was awarded a Fulbright Travel Grant. He began his graduate in the Department of Chemistry of Loyola University and was granted a Master of Science degree in February 1960. In July of 1959, he was accepted for further studies in the Department of Biochemistry.

Mr. Guschlbauer is a member of the American Chemical Society, Verein Oesterreichischer Chemiker and, since May 1961, an associate member of the Society of Sigma Xi.

In 1958, he was married to Marianna Widmann. They have one son, Wilhelm.
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Last but not least thanks are due to the author's wife, Mrs. Marianna Guschlbauer for her assistance, encouragement and patience during the progress of this research.
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CHAPTER I

INTRODUCTION

Wound Healing in History and Mythology

Even in prehistoric times, wounds and diseases received extensive attention, as attested by the skeletons dating from this period. If the beliefs and habits of present day primitive man may be used to draw conclusions about the beliefs and habits of prehistoric man, wounds were ascribed to supernatural causes in prehistoric times. Their treatment consisted of a combination of spiritualistic procedures and physical remedies. Mythology and early examples of the fine arts give evidence of interest in wounds. The famous reliefs of Nache-Rostami near Persepolis show wounded lions, the wounds being grossly enlarged and emphasized.

In Homer's "Iliad" we find various descriptions of wounds and their treatment: "He sucked out the blood (of the wound) and spread soothing drugs over it." We find frequently the desire for invulnerability expressed in the representation of such heroes as Achilles in the "Iliad". Only on his heel could he be wounded. Siegfried in the myth of the "Niebelungen",
bathed in the blood of the defeated dragon Fafner, and could only be wounded on his shoulder, where a leaf had settled during his bath. This invulnerability was desired because of the high mortality resulting from wounds.

The first document, referring to wounds is the "Edwin Smith Papyrus" (1500 B.C.) (11). It consists of a description of various remedies used in Egypt to enhance the rate of healing. These substances included fresh flesh, dressings of honey and fat etc.. We may assume that up to the time of Hippocrates no consideration was given to wounds on a more scientific level. The causes of wounds were completely ascribed to supernatural powers and it did not occur to the people of these times to investigate further.

As Greek philosophy evolved, a strong impetus was given to the natural sciences, which reached its peak in the teaching of Hippocrates (about 460 B.C.). Although primarily a physician, his approach to wound healing was experimental. As a surprising forrunner of antiseptic methods, he used hot tar in the treatment of wounds.

After Hippocrates' death, the scientific approach deteriorated. Some experimentation was carried out, but with the sole goal of finding remedies which would enhance healing, rather than explain the process involved. The approach towards wound healing after Hippocrates' time, including the Roman epoch, may be said to have been summed up in the fifth book of Celsus (30 A.D.)
This book deals exclusively with the treatment of wounds, using such drugs as myrrhe, saffron, crude potassium nitrate, iris etc.

In the medieval times, under the auspices of misinterpreted Christian philosophy, no return to Hippocratic attitude was thinkable. The idealistic, spiritualistic point of view had become prevalent. Nevertheless, out of necessity, much consideration was given to wounds. The five wounds of Christ served as the symbol of mankind's redemption in Christian mysticism. To this day many persons have been reported to have carried the "stigmata", the most famous one of all, St. Francis of Assisi (12th century). According to Christ's teaching to heal the sick, much effort was made to relieve the plight of the wounded. This helped to form an attitude, a social attitude, but one of great significance. Medieval treatment ranged from simple dressings to procedures of the strangest kind. The "powder of sympathy" was warmly recommended. It consisted of crude copper sulfate and was applied - not to the wound - but to the weapon which had caused the wound. This method was frequently successful, for the wound not being disturbed healed without infection.

Since venereal diseases may be accompanied by open wounds, people of the medieval period interpreted wounds as a sign of a soiled soul. A typical example is represented in Amfortas in the "Parcival" epic by Wolfram von Eschenbach (12th century), who suffered from an ugly wound as a punishment for his impurity.
The wound was considered to be a curse of immodesty and unchastity. Another example of medieval literature refers to the relief of the suffering caused by wounds of the plague; the hero of "Der arme Heinrich" by Hartmann von der Aue is healed by the heart blood of a pure maiden.

The entire outlook on disease was veiled by beautiful, but in a practical sense useless, interpretations of religious doctrine. Though not formally forbidden, physiological experimentation, as recommended by Hippocrates, and dissection was thus impossible. The human body as the shrine of the soul, redeemed by Christ's wounds and blood was held sacred. However, some experimentation was carried out in the empirical sense. Some scholars regarded suppuration as an essential part in the course of healing of wounds; others disagreed. Simple dressings were favored by some, a few recommended dry treatment of wounds (Henri de Montville, 13th century).

With the dawn of the great Renaissance movement and the reevaluation of Greek philosophy, the natural sciences began to bloom again. William Clowes' "Profitable Booke of Observations" (19) summarized the most usual procedures in treating wounds.

The controversial Philippus Aureolus Theophrastus Bombastus von Hohenheim, popularly called Paracelsus (1493 - 1561), a Salzburg physician, far ahead of his time, indicated, though in a somewhat obscure manner, the biochemical nature of diseases. He pointed
out nature's method of healing wounds by sealing the edges of the wound with a natural balsam (possibly fibrin clot), produced by the body. Even though he and others referred to the importance of chemistry in medicine, full recognition came only in the last century and fostered many of modern civilisation's achievement. Astonishingly enough, in the field of wound healing no consideration has been given to its chemical aspects until very recent times.

In essence the idea of enhancing wound healing by topical application of various substances has been the experimental approach to the problem from the times of Hippocrates to the twentieth century. To this time physicians are more interested in curing wounds than in understanding the processes involved. It might be pointed out that even two millennia were still insufficient to make even well educated and well trained people abandon the magical techniques of pre-Christian times.

It was not until the publication of the papers by Harvey and Howes (40, 52, 53) around 1930 that a modern biochemical approach to the study of the healing of wounds began.

Early work on wound healing

Despite the great interest in the healing of wounds since ancient times, the problem was approached from a scientific viewpoint only relatively late.
Garro1 (13) performed a series of wound healing experiments on dogs at the begin of this century at the Rockefeller Institute. He speculated on the relation between wound size and growth rate of the wound edges and defined four distinct periods during the healing wounds:

1) The "quiescent period", following the infliction of the wound lasts for one to three days. During this time the appearance of the wound barely changes.

2) The period of "granular retraction" which covers approximately the time between the third and the tenth day after wounding. During this period the wound gradually fills with granulation tissue. The edges of the wound contract significantly. Around the tenth day the contraction stops.

3) The "epidermization period" follows. It is characterized by the formation of a skin-like structure and lasts for various periods (up to several weeks).

4) During the "cicatricial period" the regenerating wound tissue is transformed into non-proliferating skin.

Despite these early attempts to characterize the healing process and the demonstration of Clark (18) that high-protein diet has beneficial effects on the healing rate, it took several years until further meaningful investigations were conducted. Harvey and coworkers (140) showed that a protein diet stimulated fibroplasia, i.e. the period of epidermization. They also showed (53) that complete starvation will retard fibroplasia
and final healing of the wound. All of these early investigations were hampered by the fact that no reproducible and reliable methods for measuring the rate of wound healing were available. The method of Howes (52) for measuring tensile strength of wound tissue was too inexact. Only since the apparatus, devised by Charney et al. (17) has been used widely, could measurements in different laboratories be reproduced satisfactorily.

Localio and coworkers (68) reformulated the stages of the healing process. This characterization is now generally accepted.

1) The phase of "traumatic inflammation", in which hyperaemia and exudation occurs.

2) The phase of "destruction", in which the wound is freed of dead and dying cellular elements. Several enzymes have recently been found to accomplish this task. Dumont (25) showed the presence of deoxyribonucleases, which eliminate DNA from nuclear material, brought in by invading leucocytes. Zamecnick et al. (142) demonstrated a strong peptidase activity in serum shortly after burns. Recently Raekallio (95) followed similar lines and demonstrated histologically the presence of leucine aminopeptidase, acid and alkaline phosphatase activity as early as four hours after wounding. These two periods constitute the so-called lag-phase of wound healing.

3) The phase of "proliferation" in which the formation of fibrous tissue occurs. This phase has also been referred to
as the period of fibroplasia, contraction or epidermization.
It is the phase of collagen deposition.

Most of these early investigations were performed using histological techniques. Johnson and McMinn (59) recently reviewed these studies extensively.

Proteins in Wound Healing

Several reviews have been published on the role of proteins in wound healing (27, 107, 128, 129).

The higher nutritional requirements of injured animals for proteins had been discovered relatively early (13). Localio et al. (69, 70) found that methionine was very effective in bringing about the normal rate of wound healing in protein depleted animals. This has been confirmed by various investigators using histological (93), nutritional (17) and chemical methods (130). Williamson and Fromm (131, 133) also showed that cystine was effective in substituting for methionine, but that ethionine and alanine could not fulfill the requirements (33). There seems to be a linear interrelationship between cystine content and tensile strength of the wound tissue (131). Also a similar linearity seems to exist with the collagen content as expressed by the hydroxyproline concentration (26, 37). However, feeding of hydroxyproline does not increase the rate of healing or the tensile strength of the wound tissue in protein depleted
rats (92). While this might seem surprising in view of the increasing amounts of hydroxyproline found in the wound tissue, several investigators discovered that hydroxyproline is not incorporated into collagen (77, 97, 98). Despite several careful investigations regarding the formation of collagen (54-57, 85, 86) the mechanism of the appearance of hydroxyproline in collagen is unknown (77).

Various other amino acids have been tested for their effects on wound healing: tryptophane, valine, lysine (78) and histidine (134). All were found to be ineffective in stimulating the rate of wound healing.

Wounding and trauma in general has profound effects on the protein metabolism of the whole injured organism. The excessive requirement of the wound for sulfur amino acids was found to be met mainly by the liver (33, 133). The sulfur amino acids are also mobilized from kidney and muscle tissue (34, 35). Probably methionine is not directly utilized but converted into cysteine (34, 35, 82, 133). These studies also showed that two different types of protein are formed during the course of wound healing: up to the eleventh day after wounding wound proteins contain more methionine, while later on the wound tissue proteins appear to contain more cystine.

A considerably higher urinary excretion of sulfur occurs in wounded animals as compared to that in normal animals (132).
By applying paper chromatography Williamson and Fasemann (135) were able to show that most of the sulfur excreted is in the form of taurine, a metabolite of cysteine. However, when calculated on the basis of excreted nitrogen, the sulfur excretion of wounded animals was lower than that of unwounded controls, thus showing a net retention.

Nucleic Acids in Healing Wounds

The formation of regenerating wound tissue involves the active formation of new cells; this infers the active synthesis of nucleic acids. As pointed out by Tremolières and Derache (118) there exists virtually no information on the amount of nucleic acids present in healing wounds, nor about the catabolism of these compounds which are necessary for regeneration, growth and differentiation. This is even more surprising since nucleic acids were originally isolated from exudates of wounds (76).

The very limited information on nucleic acids in healing wounds has been derived mainly by histological methods. Yafeleva (136) found slight increases in RNA concentration two days after wounding. Barakina (4) also found an increase in RNA concentration during limb regeneration in amphibia. Shamrai et al. (132) and Belaieff (5) showed a similar increase after infliction of burns.

Izanev and his coworkers accumulated a considerable amount of evidence in support of these ideas. These investigators
observed extensive disaggregation of RNA from its protein complexes shortly after wounding (122, 124). Breakdown of RNA is inhibited and the RNA is retained during the early phases of wound healing (119). An overall increase of RNA has also been observed in the wound (119). The disaggregation products of RNA also increased the phagocytic activity of leucocytes (120, 125). From these results Tsanev derived a complicated system of the mechanism of wound regeneration, which involves the formation of "Free RNA"; this moiety may have some relationship with s-RNA (123). From the formation of RNA-breakdown products this author also proposed a physiological mechanism involving the stimulation of subcortical centers and release of a neurohumoral factor, which in turn increased tissue reactivity (121).

It has been reported that hydrolysis products of nucleic acids stimulate the regeneration processes. They appear to exhibit a stimulating effect on the phagocytic activity of leucocytes (120, 125), but apparently also have other effects on the healing process, possibly by stimulating oxidative phosphorylation (4). Palladina and Gudina (87) found that purine containing substances are especially active in increasing the rate of wound healing. AMP has been found to be very effective in decreasing the lag phase of wound healing (96). It also has been reported that dried pancreatic preparations are very effective in increasing the rate of wound healing (104). Pancreas contains considerable amounts of enzymes which degrade nucleic
The apparent growth stimulating effect of tissue homogenates containing nucleic acids was reviewed by Paschkes (89). Paschkes and coworkers (1) and Friedrich-Freksa and Zaki (32) found that blood serum from hepatectomized rats caused stimulation of liver mitoses during liver regeneration. Teir et al. (112) found growth stimulation of homogenates of outer orbital glands on regenerating liver and other growing tissues (111). Paschkes et al. (91) also showed that a non-specific general growth stimulation takes place in hepatectomized rats. These investigators (90) also demonstrated increased growth of Walker 256 hepatoma in hepatectomized rats. On the other hand several growth inhibitors seem to be present in these tissues (36, 61, 106). There also appears to be a protecting effect against x-ray irradiation connected with the presence of nucleic acids (60, 84).

While relatively little is known about the metabolism of nucleic acids in healing wounds, a large amount of information has been accumulated on nucleic acid formation and turnover in regenerating liver. Studies with $^32P$ showed an extensive incorporation of this isotope into both RNA and DNA. While DNA seems to be metabolically very stable (28, 31, 53, 73, 83, 127), RNA is found to be formed and metabolized relatively rapidly. Similar results were obtained using $^{14}C$-labelled orotic acid (10, 42, 43, 110). The metabolic stability of DNA has also been tested
in embryonic tissue (7), in young animals (109), in lymphatic cells (137), in bacteria (105) and in tissue cultures (108, 113).

Only a few reports exist on the presence of DNA in healing wound tissue. Sherry and coworkers (103, 114) found that deoxyribonucleoproteins were significant constituents of pleural exudates, thus confirming the early findings of Miescher (76). Dumont (25) showed that most of the DNA disappeared from the wound tissue within 4.8 hours after infliction of the wound. He also found that deoxyribonucleases enhanced this process. He left open the question on the origin of the DNA in the wounds, but suggested several possibilities, such as leucocytic invasion, necrotic tissues and possibly blood vessels.

**Nucleic Acids and Protein Biosynthesis**

Several theories have been proposed to explain the mechanism by which amino acids are brought into the particular sequence which is characteristic of specific proteins (15, 24, 41). These schemes assumed the existence of "templates" which were composed of nucleic acids; the order of the nucleotides determined the order of the amino acid sequence in the protein. These theories proposed that the template must directly interact chemically with the amino acid to be ordered on its surface.

More recently an alternative hypothesis has been put forward which differs in principle and detail from the earlier
schemes. It was discovered that two distinctly different kinds of RNA - the so-called soluble or transfer RNA, and the ribosomal or particulate RNA - were involved in protein biosynthesis (8, 16, 20, 45, 48, 49, 71, 79, 115, 116). It was postulated that amino acids, after being activated enzymatically by ATP, were transferred to soluble RNA (s-RNA) molecules and that these latter molecules then brought the amino acids to the template (ribosomal RNA), reacting specifically with the template by hydrogen bonding between complementary bases of the two RNA moieties.

It is assumed that the ribonucleoprotein particles of the cytoplasm (ribosomal RNA) are the chief site of cellular protein biosynthesis and that the RNA of these particles is a template upon which the protein is formed. The following scheme has been proposed for the formation of proteins (48, 141):

1) \( aa + ATP + \text{activating enzyme} \rightarrow (aa-AMP-enzyme) + PPi \)

2) \( (aa-AMP-enzyme) + s-RNA \rightarrow (s-RNA-aa) + AMP + \text{enzyme} \)

3) \( (s-RNA-aa) + \text{enzyme} + \text{RNP} \rightarrow \text{RNA-}aa + s-RNA + \text{enzyme} \)

After activation of the amino acids by ATP (Step 1) these aminoacyl moieties are transferred directly to uniquely reactive fractions of cellular RNA (s-RNA or transfer RNA) (44, 140) (Equation 2). This RNA has a molecular weight of 20,000 to 50,000 (in contrast to the much higher molecular weight of the particulate RNA which is approximately 1,000,000). Each amino
acid requires a specific soluble RNA molecule. The amino acid is esterified on the 2' or 3' hydroxyl group of the terminal adenosine (39, 47, 94, 140). Partial fractionations of individual transfer RNAs have been accomplished (2, 3, 12, 51, 65).

Since each amino acid requires a specific s-RNA molecule each reaction is catalyzed also by a specific enzyme. These enzymes are probably identical with the activating enzymes, responsible for the initial formation of the acyl-adenylate-enzyme complex (aa-AMP-enzyme in Equations 1 and 2).

The amino acid - s-RNA complex then transfers its amino acid to the appropriate position on the particulate RNA (Equation 3). This position is dependent upon the sequence of nucleotides in the microsomal RNA (50, 141). Incorporation of amino acids into protein can be accomplished with activating enzyme, soluble RNA and particulate RNA from three different sources, even of different species. The protein formed will be determined by the source of the microsomal RNA (72, 141). While most of these studies were performed in vitro, recent investigations in vivo systems are in full agreement with these theories (49, 63).
CHAPTER II

EXPERIMENTAL PROCEDURES AND METHODS

A) DESIGN OF EXPERIMENTS

The goal of this dissertation was to elucidate some of the relations of the nucleic acids in healing wounds. There is no report in the literature on the levels of these compounds in wound tissue (118). It was therefore considered necessary to begin the study with an extensive analysis for RNA and DNA in wound tissue. Simultaneously the influence of wounding on the nucleic acid levels of liver and kidney was studied.

Experiment #1

White female adults virgin Sprague Dawley albino rats were individually caged and fed the protein-free diet described below. After four days the animals were wounded according to the usual procedure, described on page 19. The animals were kept on the protein-free diet throughout the experiment. Several animals were not wounded but immediately sacrificed and the kidney and liver was removed. These samples served as the controls.

At intervals of 1, 3, 5, 8, 11, 15, and 19 days after wounding several animals were sacrificed, the wound tissue harvested
and the kidneys and livers removed. Wound tissue was collected only from the fifth day on, since insufficient tissue was grown at earlier dates. The tissue samples were fractionated and analyzed for pentoses and phosphate in the RNA, DNA and acid soluble phosphate (ASP) fraction.

Experiment # 2

Experiment # 2 was performed in the same way as experiment # 1 with some minor changes. Wound tissue, liver and kidney samples were taken at 4, 6, 9, 11, and 17 days after wounding, while four unwounded rats were sacrificed on the day of wounding as controls for the kidney and liver determinations. The samples were fractionated and analyzed as in the previous experiment.

Experiment # 3

This experiment was designed to confirm and expand the information obtained in the first two experiments. To investigate the rate of formation of individual nucleic acid fractions and to elucidate the changes in the non-regenerating tissues tracer studies with P$_{32}$ were undertaken. The procedure for this experiment was identical with that in experiments # 1 and # 2. The animals were sacrificed at 5, 8, 12, and 15 days after wounding. Two hours before sacrificing each rat received an intraperitoneal injection of 80 µC of sodium phosphate-P$_{32}$. Again wound, liver
and kidney samples were collected. The samples were homogenized and fractionated as described below. From each fraction aliquots were withdrawn and dried in stainless steel planchets. The radioactivity was determined in a Geiger-Müller end-window counter.

Experiment #4

In this experiment the metabolic fate of nucleic acids was followed by measuring the change of uptake of P^{32} with time. Three groups of rats were maintained and wounded as in the previous experiments. The first group received 30 uC P^{32} per rat on the fifth day after wounding. Several animals were then killed at 4, 8, 16, 24, and 48 hours after the administration of the radioactive tracer. Only the wound tissue was harvested in this experiment. Each sample was analyzed for phosphorus and radioactivity. The same procedure was carried out on the seventh and eleventh day after wounding on the remaining two groups of animals.

Experiment #5

This experiment was performed to get an insight into the relationships between nucleic acids and protein formation during experimental wound healing. An analysis for the characteristic amino acids was therefore of interest. Rats were maintained and
wounded in the usual way. After 5, 7, 12, and 18 days groups of rats were sacrificed, their wound tissue collected and weighed into screw-cap tubes. After adding 5.0 ml 5 N NaOH, the tubes were covered with Parafilm and closed tightly with the screw caps. The tubes were then heated in a covered boiling water bath for six hours. During this time no liquid had evaporated. The tubes were filled to 10.0 ml volume and analyzed for tyrosine, hydroxyproline, and total nitrogen.

In addition the protein residues, after removal of the nucleic acids, from the previous experiment were hydrolyzed and analyzed for tyrosine.

B) EXPERIMENTAL PROCEDURES

Experimental Animals

In all experiments to be reported in this dissertation female albino rats of the Sprague Dawley strain were used. The animals in any experiment were of about equal weight, generally about 180 ± 20 grammes. Mature female rats were used because they maintained constant weight under normal feeding conditions, thus eliminating growth or senility factors.

The rats were housed in metabolism cages and were weighed at regular intervals during the course of each experiment.

Wounding Procedure

After an acclimation period of two to four days on the
protein-free diet the animals were given an injection of sodium nembutal intraperitoneally (3.5 mg/100 g body weight). Ether was used to anesthetize the animals completely.

After removal of most of the hair on the back of the neck, the outline of a coin (4 cm in diameter) was traced and the skin was excised down to the muscle along the outline. The wounds were allowed to heal without any further attention. Only one case of infection was observed in over 150 animals used.

**Diet**

In all experiments reported the animals were fed a standard protein-free diet. Thus the experimental animals supplied their own nitrogen sources for protein and nucleic acid synthesis. The diet contained the following ingredients:

- 1620 g sucrose
- 200 g lard
- 40 g vitamin mixture *)
- 80 g HMW salt mixture *)
- 20 g Alpha Cell *)
- 40 g cod liver oil

2000 g

The vitamin mixture was ground in a mortar with increasing

*) Supplied by Nutritional Biochemical Corp. Cleveland Ohio.

For the composition see Appendix I.
amounts of sucrose until all the vitamins were equally distributed. The salt mixture and the Alpha-Gel were intermixed with the sucrose-vitamin mixture with a spoon. Meanwhile the lard was molten and mixed with the cod liver oil. The fat mixture was poured into the sugar mixture and stirred well with a spoon. The diet was stored in a refrigerator to prevent the possibility of rancidity.

Each animal received 8 grammes of this diet per day, an amount which was used completely prior to the next feeding period. Water was allowed ad libitum.

Harvesting of the Tissues and Homogenization

At designated days (usually 5, 8, 12, and 15 days after wounding the rats were anesthetized with nembutal and ether as for the wounding procedure. The scab of the wound was removed with forceps and the wound was cleaned superficially with absorbant paper, to remove scab and hairs. With scissors the tissue was cut along the outline of the wound and normal skin. After the wound was excised the remaining connective tissue was removed as carefully as possible. The wound tissue was placed into a weighing bottle, frozen, weighed and transferred to a chilled mortar. A spatula of sand (approximately 0.5 grammes) was added and the tissue was homogenized with the pestle as rapidly as possible. After the tissue was grossly disintegrated
several milliliters of water or physiological saline were added and the homogenization continued. Repeated extraction with water or saline was continued until the sand was essentially free of tissue fragments. The homogenate was made to volume, an aliquot for total nitrogen determination was removed and the rest was used for the further purification and fractionation of the nucleic acids.

In some cases also liver and kidney samples were taken from the animals together with the wound tissues. In the first experiment the kidneys and livers were homogenized in a chilled teflon homogenizer, a method which was later abandoned. The non-regenerating tissues were homogenized with sand in the same fashion as were the wound tissues. The two methods of homogenization of the non-regenerating tissues appeared to make no difference in the analytical results, as will be seen in the data from experiments # 2 and # 3.

Separation of Nucleic Acids.

The method used for the separation of the nucleic acids is a modification of the method of Schmidt and Thannhauser (100) and Schmidt (101). It consists mainly in the precipitation of proteins and nucleic acids with trichloracetic acid (TCA) and further separation by fractional alkaline hydrolysis. The homogenates were mixed with an equal volume of 10 percent TCA in
the cold, which resulted in the precipitation of proteins and nucleic acids; the supernatant contained all the acid soluble components of the cell, such as sugars and their derivatives, amino acids, purine and pyrimidine derivatives of low molecular weight, organic acids, etc.. The phosphorus determined in the supernatant fraction is commonly called "acid soluble phosphorus" (ASP). The precipitate was separated by centrifugation in the cold. The precipitate was washed twice with 5 percent TCA to remove traces of soluble material and the washings were combined with the first supernatant.

The protein-nucleic acid precipitate was now suspended in 5 ml 80 percent ethanol, which dissolved most of the lipids of the precipitate (from phospholipids, lipoproteins and neutral fats) and the suspension was spun down in the cold. The supernatant was discarded and the precipitate was washed twice more with ethanol, once with ethanol-ether (1:1), and twice with ether. After drying the precipitate, it was incubated for approximately 18 hours at 37° C with 5.0 ml 1 N KOH. This dissolved the precipitate completely. This treatment breaks RNA into mono- and dinucleotides, but DNA and proteins are only denatured by this procedure. Addition of 0.5 ml 8 N HCl and of 5.0 ml 10 percent TCA precipitated DNA and protein, while the RNA fragments stayed in solution. The nucleic acid fractions were then separated by centrifugation. The precipitate was
washed twice with 5 percent TCA in the cold; the supernatant solutions were combined and were called the "RNA fraction". Observations by Davidson and coworkers (21, 22) had shown that small amounts of orthophosphate can be found in this fraction; these arise largely from the alkaline hydrolysis of phosphate from phosphoproteins. It was found similarly that orthophosphate was present in amounts between 0.5 and 3.0 percent of the RNA phosphorus. The amounts of RNA phosphorus reported in this dissertation are not corrected for this discrepancy.

<table>
<thead>
<tr>
<th>Wound sample #</th>
<th>Percent inorganic phosphate in RNA fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.11</td>
</tr>
<tr>
<td>2</td>
<td>2.31</td>
</tr>
<tr>
<td>3</td>
<td>1.84</td>
</tr>
<tr>
<td>4</td>
<td>3.02</td>
</tr>
</tbody>
</table>

The precipitate, which contained DNA and denatured protein was now treated with 5.0 ml 5 percent TCA for 15 minutes in a boiling water bath. After cooling the tubes were centrifuged, the supernatant saved, and the precipitate treated again with hot TCA. This procedure was repeated twice. The supernatant solutions were pooled and were considered to be the "DNA fraction". The residue contained the proteins. It was discarded or hydrolyzed in hot alkali for the determination of tyrosine.
C) ANALYTICAL PROCEDURES

Nitrogen (Micro-Kjeldahl)

The nitrogen analyses reported were obtained by the micro-Kjeldahl method. An aliquot of the tissue homogenate or an unhomogenized weighed piece of tissue was placed into an 8 inch test tube and was digested with 2 ml 9 N sulfuric acid. After blackening of the residue three drops of 30 percent hydrogen peroxide were added and the heating continued until white fumes of sulfur trioxide appeared. The test tubes were cooled and approximately two milliliters of distilled water were added and evaporated on the sand bath and on the microflame until fumes of sulfur trioxide appeared again. This procedure converted the tissue nitrogen into ammonium sulfate. The digest was then cooled and diluted to 10.0 ml. Aliquots of this solution were transferred to a micro-Kjeldahl distillation apparatus. By the addition of 50 percent sodium hydroxide the ammonium ions were converted to ammonia gas, which was steam-distilled into an aliquot of standardized sulfuric acid. The residual acid was back titrated with standardized base, using brom cresol green as the indicator. From the difference of these volumes the amount of nitrogen was then calculated.
Phosphorus

The method of Fiske and SubbaRow (29) in the modification of Leloir and Cardini was used to determine the total phosphorus content of the different fractions isolated.

Sample aliquots and standards were placed into 8 inch test tubes, and 1.0 ml 5 N sulfuric acid was added. The samples were digested on a sand bath and over a microflame until coloration occurred. The tubes were allowed to cool and 0.2 ml concentrated nitric acid was added to each tube. The heating was continued until white fumes of sulfur trioxide appeared. The samples were cooled again, approximately two milliliters of distilled water was added, and heated again until fumes of sulfur trioxide appeared. The samples were then washed quantitatively into 10.0 ml volumetric flasks and placed into a boiling water bath. To each flask were added 1.0 ml 2.5 percent ammonium molybdate solution and 0.2 ml of Reducing Reagent solution. The flasks were then allowed to cool slowly to room temperature and the blue color was measured in a Klett-Summerson colorimeter with a # 60 filter.

The Reducing Reagent was prepared by dissolving 0.25 g of the following mixture in 10.0 ml distilled water: 1.2 g sodium sulfite, 1.2 g sodium bisulfite, 0.2 g 1-amino-2-naphthol-4-sulfonic acid. The three chemicals were mixed well in a mortar before dissolving.
Ribose

The method of Nejbaum (75) was used to measure ribose. This method determines only ribose bound to purines (126), since the ribose pyrimidine bond is not broken under the experimental conditions used.

Standards and samples were placed into test tubes and filled with distilled water to a total volume of 5.0 ml. When the samples were in trichloracetic acid solution, the standards were filled to 5.0 ml volume with 5 percent TCA. 5.0 ml of the reagent (0.7 g orcinol and 0.2 g FeCl₃ in concentrated HCl) were added with shaking. The samples were then heated for 30 minutes in a boiling water bath. After 30 minutes cooling the color was measured in a Klett-Summerson colorimeter with a # 66 filter.

Deoxyribose

The method used for the quantitative measurement of deoxyribose was a modification (101) of the original method of Dische (23). This method too measures only purine-bound deoxyribose.

Samples and standards were placed into test tubes and filled to 2.5 ml volume with distilled water or 5 percent TCA. Five milliliter of the reagent (1.0 g diphénylamine in 100 ml glacial acetic acid plus 2.75 ml of concentrated sulfuric acid) were added and the tubes immersed into a boiling water bath for
15 minutes. After cooling for 10 minutes, the absorption was measured in a Klett-Summerson colorimeter with a #60 filter.

**Hydroxyproline**

The following method proved to be the most suitable for the determination of hydroxyproline. It is a modification of the method of Neumann and Logan (80, 81).

One milliliter aliquots of the unknown samples and standard solutions were pipetted into a 6 inch test tubes. One ml of 2.5 N NaOH, 1.0 ml 0.005 M copper sulfate solution and 1.0 ml 6 percent hydrogen peroxide were added. The tubes were then immersed into a water bath at 50°C as recommended by Leach (64). After 15 minutes the tubes were removed and cooled in an ice bath. After cooling, 0.2 ml of M/20 ferrous sulfate solution was added to each tube to remove traces of hydrogen peroxide (74); the mixture was then shaken well and 4.0 ml 3 N sulfuric acid added. With shaking, 2.0 ml of the developing reagent (5 percent N-dimethylamino benzenaldehyde in n-propanol) were added. The tubes were immersed into a water bath at 60°C for 15 minutes. After this period the tubes were cooled in ice water and the extinction determined in a Klett-Summerson colorimeter with a #54 filter.

Note: It was necessary to hydrolyze the tissue in 5 N base under pressure for 6 hours, since acid hydrolysis gave low hydroxyproline values. Adjustment of the base present had to be made.
Tyrosine

The method of Folin and Ciocalteau was used (30).

One milliliter standards and sample aliquots were placed into test tubes; 10.0 ml water was added to each tube and the tubes well shaken. One half milliliter of 2.5 N NaOH and 2.0 ml 1 N Na$_{2}$CO$_{3}$ solution were added and the content of the tubes was shaken well; 2.0 ml of the Folin-reagent were added and the tubes were shaken immediately after addition vigorously. After 20 minutes the absorption was determined with a #66 filter in a Klett-Summerson colorimeter.

It was found that this method determined about 90 - 95 percent of the tryptophane present (88).

Folin Reagent: 100 g sodium tungstate and 25 g sodium molybdate were dissolved in 700 ml distilled water, 50 ml 85 percent phosphoric acid and 100 ml concentrated hydrochloric acid was added. The mixture was refluxed for about 10 hours. After cooling 150 g lithium sulfate, 50 ml distilled water and about 10 drops of liquid bromine were added. This solution was now boiled for 15 minutes without condenser. After cooling the solution was diluted to one liter and filtered through glass wool.

Determination of Radioactivity

For the measurement of radioactivity in samples, essentially conventional methods were used. Aliquots of the samples
in question were plated on stainless steel planchets and equally distributed with distilled water or a drop of Duponol. The samples were then dried under an infrared lamp.

In the first experiment involving radioactivity (Experiment # 3) a Geiger - Müller end window tube (window thickness: 1.7 mg/cm²) was used with a RCL counter (Radiation Counter Laboratories, Scaler Mark 13, Model I). In all later experiments a gas flow assembly was used with a Tracerlab Autoscaler.

All the radioactivity data reported were corrected for decay.
CHAPTER III

RESULTS AND DISCUSSION

Since no information has been published on the levels of RNA and DNA in regenerating wound tissue, the first three experiments were devoted to determine the concentration of these compounds in wound tissue. Simultaneously the effect of wounding on nucleic acid metabolism in liver and kidney was investigated.

Levels and Metabolism of Nucleic Acids in Healing Wounds

In two independent experiments using 40 rats each, the concentration of RNA, DNA and acid soluble phosphorus (ASP) was determined. The levels of these compounds were also determined in liver and kidney tissue. The rats were maintained on the protein-free diet and wounded by the standard procedure described above. The samples were harvested at intervals between the fourth and nineteenth day after wounding and fractionated as described. The individual fractions were analyzed for phosphate (29) and sugars (75, 101).

Experiment # 3 was similar to the first two experiments. It was found desirable to check and supplement the information
Fig. 1: The DNA content of regenerating wound tissue in micromoles of nucleic acid phosphorus per milligram of tissue nitrogen at various intervals after wounding. The data from the first three experiments are included. Each point represents the data from analysis of tissues of 4 to 6 rats. The vertical lines through the data points show the standard deviation.
of the first two experiments. The third experiment involved, in addition to the determination of nucleic acid concentration, the use of $^{32}P$ as a tracer for the study of the rate of biosynthesis of the nucleic acids. Each of the animals used in this experiment received 80 µg sodium phosphate-$^{32}P$ two hours before sacrifice.

The amount of DNA per milligram tissue nitrogen contained in regenerating wound tissue at various intervals after wounding is shown in Fig. 1. The data from all three experiments are included. Since very little wound tissue is formed prior to the fourth day after wounding, no tissue was collected before this date. The level of DNA per mg of tissue nitrogen appears to increase for about eight days and thereafter gradually decreases. This decrease may be more apparent than actual, since the nitrogen content of this tissue has been shown to increase as regeneration progresses (26, 128, 133). Thus on the basis of tissue weight the DNA seems to remain almost unchanged after once it has reached the maximal level. This seems to indicate that the formation of new cells as indicated by the synthesis of DNA is virtually stopped by about the tenth day after wounding. If DNA formation would still continue a mechanical or enzymatic removal of part of the "old" DNA must be assumed.

The concentration of RNA per mg of tissue nitrogen in the regenerating wound tissue is shown in Fig. 2. It also reaches
Fig. 2: The RNA content of regenerating wound tissue in micromoles of nucleic acid phosphorus per milligram of tissue nitrogen at various intervals after wounding. The data from the first three experiments are included. Each point represents the data from analysis of tissues of 4 to 6 rats. The vertical lines through the data points show the standard deviation.
the highest level at about the eighth day after wounding. At this time there is more than twice as much RNA as DNA, on a molecular basis, present in the regenerating tissue. However, after the eighth day the RNA/nitrogen concentration decreases sharply. To some extent this very great decrease in RNA level, is, as in the case of DNA, a reflection of the increasing nitrogen content of the regenerating tissue. However, even on the basis of tissue weight, there appears to be an appreciable decrease in the amount of RNA in the regenerating wound tissue from the eighth day after wounding on.

Table I shows the amount of radioactive phosphorus incorporated into the nucleic acids of regenerating wound tissue two hours after administration of the radioactive label. The incorporation of $^{32}$P into the RNA fraction indicates a rapid production of this nucleic acid during the early stages of tissue formation. Thereafter the rate of RNA formation decreases very considerably to a much lower level, which, however, remains relatively constant. This reduced rate of RNA formation could be another factor responsible for the decreased level of RNA in wound tissue in the later phases of the healing process, as shown in Fig. 2.

In terms of specific activity the most rapid formation of DNA, which is indicative of the greatest mitotic activity and cell proliferation (108) appears at about the eighth day after
### Table I

**INCORPORATION OF $^{32}$P INTO THE NUCLEIC ACIDS OF REGENERATING WOUND TISSUE**

<table>
<thead>
<tr>
<th>Days After Wounding</th>
<th>RNA</th>
<th>DNA</th>
<th>ASP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Counts / Minute / μMoles Phosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>940 ± 100</td>
<td>230 ± 25</td>
<td>3560 ± 310</td>
</tr>
<tr>
<td>8</td>
<td>260 ± 100</td>
<td>720 ± 160</td>
<td>2880 ± 370</td>
</tr>
<tr>
<td>12</td>
<td>240 ± 100</td>
<td>235 ± 45</td>
<td>3290 ± 640</td>
</tr>
<tr>
<td>15</td>
<td>190 ± 75</td>
<td>100</td>
<td>7480 ± 680</td>
</tr>
</tbody>
</table>
wounding. However, within two weeks the uptake of $^{32}$P into the DNA fraction becomes practically zero. In this way the regenerating wound tissue eventually assumes the characteristics of a non-regenerating tissue with respect to production and turnover of DNA (31).

The specific activity of the acid soluble fraction, which contains not only nucleotides but also other organic and inorganic phosphates of low molecular weight, remained approximately unchanged throughout almost the entire experimental period. The great increase at about 15 days may be a reflection of the fact that relatively little RNA and essentially no DNA formation is observed at this time. A greatly reduced phosphate utilization seems to be likely and an increase in the specific activity of the ASP fraction might be expected.

Changes in the Base Composition of RNA during Wound Tissue Regeneration

It is known that the orcinol method (75) measures only the ribose bound to purines (126). On the other hand the phosphate method (29) determines the total phosphate present. By determining the nucleic acid composition with these two methods the relative base composition may be determined. As in DNA the purine/pyrimidine ratio of RNA is approximately 1. There are, however, reports of significant deviations of this equality of
**Table II**

**PURINES AND PYRIMIDINES IN RNA**

**OF REGENERATING WOUND TISSUE**

<table>
<thead>
<tr>
<th>Days After Wounding</th>
<th>μMoles Ribose per mg Tissue N</th>
<th>μMoles Phosphate per mg Tissue N</th>
<th>Purines* Pyrimidines</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.25 ± 0.08</td>
<td>0.46 ± 0.11</td>
<td>1.11 ± 0.12</td>
</tr>
<tr>
<td>8</td>
<td>0.60 ± 0.11</td>
<td>1.16 ± 0.12</td>
<td>1.05 ± 0.07</td>
</tr>
<tr>
<td>12</td>
<td>0.41 ± 0.09</td>
<td>1.01 ± 0.11</td>
<td>0.70 ± 0.07</td>
</tr>
<tr>
<td>15</td>
<td>0.33 ± 0.04</td>
<td>0.88 ± 0.12</td>
<td>0.61 ± 0.09</td>
</tr>
</tbody>
</table>

* The standard error was calculated from the purine/pyrimidine ratios for individual tissue samples.
purines and pyrimidines, especially in s-RNA(117).

If the types of RNA molecules synthesized by the cells can be shown to change it might be considered to be indirect evidence that new or different proteins are synthesized. A change in the type of RNA, or the variation in the ratio of different types of RNA produced by the cell, might be signaled by changes in the purine/pyrimidine ratios.

To investigate, if such changes might take place, the RNA samples from experiment # 3 were analyzed by the two methods mentioned above, ribose by the orcinol method (75) and phosphate according to Fiske and SubbaRow (29). The results of this experiment are presented in Table II. It appears that up to the eighth day after wounding the purine concentration is about equal to that of the pyrimidines. In the later part of the healing period, more pyrimidines than purines are found in the wound tissue RNA. This may be interpreted to mean that different types of RNA are synthesized at different stages of the healing process. A change in the nature of the proteins seems to be probable. However, large amounts of collagen appear only after the eighth day after wounding (26). Since the amino acid composition of collagen (and probably that of its intracellular precursor also) is very different from that of metabolically active proteins, it does not seem improbable that the nucleotide composition of the RNA possibly involved in the synthesis of the
precursor might be significantly different. It seems probable, however, that some of the metabolically active proteins are still formed. The nucleotide composition of a kind of RNA, specifically related to collagen biosynthesis, might be expected to show even greater differences than is indicated by the above data.

**Effect of Wounding on Nucleic Acid Levels and Nucleic Acid Metabolism in Non-regenerating Tissues**

It has been shown that wounding has a profound influence on the protein metabolism of wounded animals (34, 35, 128). To find if this stimulus also affects nucleic acid metabolism, liver and kidney tissue samples from normal and wounded animals were fractionated and the nucleic acid components were measured. In all experiments it was found that the DNA level remained unchanged over the entire period of observation. However, the amount of RNA in these tissues showed some fluctuations. In the liver the concentration of RNA began to rise rapidly and reached a peak level in about 5 to 6 days after wounding. After a significant decrease, the RNA concentration began to rise rapidly again. The same situation was found to hold with regard to the level of RNA in the kidney with the exception that the first period of RNA deposition was so small that it was not statistically significant. These results are presented in Table III and Fig. 3.
Table III

Nucleic Acid Content of Non-regenerating Tissues of Wounded and Normal Rats

<table>
<thead>
<tr>
<th>Days After Wounding</th>
<th>LIVER</th>
<th>KIDNEY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNA</td>
<td>ASP</td>
</tr>
<tr>
<td>0*</td>
<td>1.20 ± 0.05</td>
<td>1.11 ± 0.14</td>
</tr>
<tr>
<td>5</td>
<td>1.45 ± 0.02</td>
<td>2.37 ± 0.18</td>
</tr>
<tr>
<td>8</td>
<td>1.02 ± 0.07</td>
<td>2.12 ± 0.09</td>
</tr>
<tr>
<td>12</td>
<td>1.96 ± 0.30</td>
<td>2.09 ± 0.14</td>
</tr>
<tr>
<td>15</td>
<td>2.17 ± 0.28</td>
<td>1.66 ± 0.14</td>
</tr>
</tbody>
</table>

μMoles Phosphate per mg Tissue Nitrogen

* Unwounded rats.
The uptake of $^{32}\text{P}$ by the nucleic acids of non-regenerating tissues, two hours after administration of radiophosphorus, is presented in Table IV. The specific activity of the $^{32}\text{P}$ in the RNA and acid soluble phosphate fraction (ASP) of both the liver and kidney in the wounded rats is much reduced below that found in control animals. This decreased ability to incorporate the label appears to continue until after the maximal utilization of nucleotides by the regenerating wound tissue for the formation of nucleic acids. After this time, the rate of formation of nucleotides and RNA in the non-regenerating tissues began to return to the level found in the unwounded animals.

The inhibition of $^{32}\text{P}$ uptake by the liver during the early phases of wound regeneration may be a partial explanation of the somewhat greater slope of RNA formation in liver and kidney between the 10th and 17th day as compared to the period between the 1st and 6th day after wounding. It seems possible also that the stimulus of wounding may affect the formation of nucleotides rather than the synthesis of RNA from nucleotides, since the decreased uptake of $^{32}\text{P}$ by the RNA and by the ASP fractions appears to follow the same pattern of change.

The overall increase in the level of RNA in the non-regenerating tissues may be presumed to be part of a mechanism to replace the protein which has been utilized to supply the excess sulfur amino acids required by the regenerating wound tissue.
Fig. 3: The RNA content of liver and kidney of wounded rats in micromoles of nucleic acid phosphorus per milligram of tissue nitrogen at various intervals after wounding. The data at time zero represent analyses of tissues of unwounded animals.

Solid circles: kidney, open circles: liver.
**Table IV**

**INCORPORATION OF P\(^{32}\) INTO THE NUCLEIC ACIDS**

**OF NON-REGENERATING TISSUES OF WOUNDED RATS**

<table>
<thead>
<tr>
<th>Days After Wounding</th>
<th>LIVER</th>
<th>KIDNEY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNA</td>
<td>ASP</td>
</tr>
<tr>
<td></td>
<td>Counts per Minute per μMole Phosphate</td>
<td></td>
</tr>
<tr>
<td>0*</td>
<td>1081 ± 270</td>
<td>9175 ± 1170</td>
</tr>
<tr>
<td>5</td>
<td>590 ± 140</td>
<td>5140 ± 1070</td>
</tr>
<tr>
<td>8</td>
<td>520 ± 160</td>
<td>4890 ± 1030</td>
</tr>
<tr>
<td>12</td>
<td>650 ± 200</td>
<td>5520 ± 865</td>
</tr>
<tr>
<td>15</td>
<td>695 ± 145</td>
<td>6540 ± 430</td>
</tr>
</tbody>
</table>

* Unwounded Rats
proteins and the residual nitrogen to make up the negative nitrogen balance (34, 35, 128). Since, as shown in Table IV, the formation of RNA is markedly decreased below normal during the period of wound tissue regeneration, the rising level of RNA in the non-regenerating tissues must be attributed to an inhibition in the breakdown of this nucleic acid.

Although there appears to be no significant change in the ASP fraction of the kidney, this fraction increases very rapidly in the liver after wounding and then gradually returns to the normal level. It seems possible that these changes are related to the great increase in the production of urea in the liver since they coincide approximately with the period of excessive protein catabolism and nitrogen excretion which is usually observed after injury.

Uptake of Radiophosphorus in Nucleic Acids with Relation to Time

Three groups of rats were maintained and wounded as in the previous experiments. The first group received 80 µc P³² per rat on the fifth day after wounding. Several animals were then sacrificed at 4, 8, 16, 24, and 48 hours after injection of the label. Only wound tissue was collected. Each sample was analyzed for nucleic acid phosphorus and radioactivity. The same procedure was carried out on the two other groups on the seventh and eleventh day after wounding.
Fig. 4: Incorporation of $^{32}$P into the ASP fraction of regenerating wound tissue at various intervals after wounding. The data are presented as cpm per µM ASP. Each point represents analyses from 5 or 6 animals. Solid Circles: 5 days, halved: 7 days, open: 11 days.
**Fig. 5:** Incorporation of $^{32}$P with time into the ASP fraction of regenerating wound tissue at various times after wounding. The data are presented as cpm per mg tissue nitrogen. Each point represents average analyses from 5 or 6 animals.

**Solid circles:** 5 days, **halved:** 7 days, **open:** 11 days after wounding.
Fig. 4 shows the incorporation of $P^{32}$ into the acid soluble fraction. This graph demonstrates that the peak of labeling is obtained four hours after injection of the radioisotope, i.e. equal distribution throughout the animal is reached. This is in agreement with previous observations by Sacks (99).

When the activity of the ASP fraction is calculated on the basis of tissue nitrogen instead of on the basis of acid soluble phosphorus, the curves seem to coincide at the three stages of healing and the metabolic decay curves seem to be identical (Fig. 5). Since calculation on the basis of tissue nitrogen is a measure of the concentration of radioactivity per unit time, this could be explained as indicating identity in pool size, regardless of the stage of healing. This thought seems to be compatible with the ideas presented above, since mobilization of nucleotides from liver and kidney and even breakdown of nucleic acids in these tissues was found (Fig. 3). It also suggests that the healing wound has the complete body pool at its disposal.

Incorporation of $P^{32}$ into Ribonucleic Acid of Wound Tissue

The data on the specific activity of the RNA fraction after the administration of $P^{32}$ when tissue regeneration has progressed for 5, 7, and 11 days are shown in Fig. 6. The curves in this graph indicate that both synthetic and catabolic processes take
Fig. 6: Incorporation of $^{32}$P into the RNA fraction of regenerating wound tissue at various intervals after wounding. The data are presented as cpm per micromoles RNA-phosphorus. Each point represents average analyses from 5 or 6 animals.

Solid circles: 5 days, halved: 7 days, open: 11 days after wounding.
place on the fifth and eleventh day after wounding, while on the seventh day the anabolic processes seem to predominate.

An integrated picture of RNA metabolism may be constructed from the data in Fig. 2 and Fig. 6. Let us first consider a situation wherein the nucleotides of the ASP fraction are utilized for the formation of RNA as in the following reaction scheme:

\[
\text{nucleotides} \xleftrightarrow{\frac{k_1}{k_2}} \text{RNA}
\]

(1)

It then follows that for the velocities of the two reactions:

\[
R_1 = k_1 \cdot \text{(nucleotides)} \cdot (F_1)
\]

(2)

\[
R_2 = k_2 \cdot \text{(RNA)} \cdot (F_2)
\]

(3)

where \( R_1 \) is the velocity of the forward reaction, \( R_2 \) is the velocity of the hydrolytic reaction; \( F_1 \) is the concentration of the enzymes and other factors in the synthesis of RNA, while \( F_2 \) is the concentration of the necessary enzymes and factors involved in the catabolic reaction.

From the data presented in Fig. 2 the following relationships between the reaction velocities appear to exist:

On Day 5 after wounding: \( R_1 > R_2 \)

(4)

On Day 7 after wounding: \( R_1 = R_2 \)

(5)

On Day 11 after wounding: \( R_1 < R_2 \)

(6)

However, the data in Fig. 6 show that on the seventh day after wounding RNA synthesis is greater than hydrolysis, i.e. \( R_1 > R_2 \).
Therefore Equation (1) cannot be describing the true situation with regard to the metabolism of RNA.

Another explanation may account for the data in Fig. 2 and in Fig. 6. It depends on the assumption that two different types of RNA are being formed. The "fast RNA" is formed much more rapidly than the "slow RNA" and is assumed to be metabolically more active. Therefore it will also be broken down much faster than the slower moiety. The following scheme will describe the situation:

\[
\begin{align*}
\text{RNA-f} & \leftrightarrow \frac{k_1}{k_2} \text{nucleotides} \leftrightarrow \frac{k_3}{k_4} \text{RNA-s} \\
\end{align*}
\] 

By definition:
\[
\begin{align*}
&k_1 > k_3 \\
&k_2 >> k_4
\end{align*}
\]

Transforming this mechanism into a mathematical form, the following reaction velocities are obtained:

\[
\begin{align*}
R_1 &= k_1 \cdot (\text{nucleotides}) \cdot (F_1) \\
R_2 &= k_2 \cdot (\text{RNA-f}) \cdot (F_2) \\
R_3 &= k_3 \cdot (\text{nucleotides}) \cdot (F_3) \\
R_4 &= k_4 \cdot (\text{RNA-s}) \cdot (F_4)
\end{align*}
\]

where \(R_1\) and \(R_3\) are the velocities of the forward reactions, \(R_2\) and \(R_4\) are the velocities of the catabolic reactions, \(F_1, F_2, F_3,\) and \(F_4\) are the concentrations of the enzymes and other factors involved in the respective reactions, \(k_1, k_2, k_3,\) and \(k_4\) are the rate constants of the four reactions.
From Fig. 2 the following relations may now be derived:

On Day 5 after wounding: \((R_1 + R_3) > (R_2 + R_4)\) (14)
On Day 7 after wounding: \((R_1 + R_3) = (R_2 + R_4)\) (15)
On Day 11 after wounding: \((R_1 + R_3) < (R_2 + R_4)\) (16)

For the 5th day after wounding Fig. 6 shows the following situation:

\[(R_1 + R_3) > (R_2 + R_4)\] (17)

which would be in agreement with Equation (14). But the 48 hour measurement after the isotope administration indicates that the following picture might be predominant:

\[(R_1 + R_3) \leq (R_2 + R_4)\] (18)

The last Equation (18) shows considerable breakdown 48 hours after injection of P\(^{32}\). Since RNA-s is formed much more slowly, the concentration of this moiety will be very low in comparison with the rapidly formed RNA-f. This automatically decreases the contribution of reaction \(R_4\), which is relatively slow in any case, to the breakdown. Besides this, the back reaction \(R_2\) will be more pronounced because of the faster rate \((k_2 \gg k_4)\) and the higher concentration of RNA-f. Since \(k_2 \gg k_3\) (Equation (6)), RNA-f will be formed faster, i.e. \(R_1\) is the predominating reaction on the fifth day after wounding.

While Equation (18) only demonstrates the presence of an increased relative breakdown, this may be due to several causes. It will mainly be due to the increase in concentration of RNA-f.
However, the decreased uptake of label in the RNA can also be
due to a decreased formation of RNA-f. This might be caused by
a change in the concentration in the term (F1), i.e. the enzymes
or cofactors will be at a lower level, possibly caused by a li-
miting concentration of RNA or the presence of a "negative feed-
back mechanism", as demonstrated by Yates and Pardes for pyrimi-
dine biosynthesis (138, 139).

This is in conformity with the information which appears
in Fig. 7.

Turning now to the metabolic situation of RNA at the 7th
day after wounding, Equation (15) derived from Fig. 2 will apply:

\[(R_1 + R_3) = (R_2 + R_4)\]  \hspace{1cm} (15)

Synthesis and breakdown are in equilibrium. When considering
Fig. 6, a different relation will be obtained:

\[(R_1 + R_3) > (R_2 + R_4)\]  \hspace{1cm} (19)

By the seventh to eighth day after wounding the RNA concen-
tration has reached its peak level (Fig. 2). While there is
still active RNA-s synthesis (the back reaction R_4 probably never
amounts to very much), the synthesis of RNA-f is markedly de-
creased. This decrease is possibly due to an inhibiting effect
of a limiting concentration or a "negative feedback mechanism"
on the enzymes (F1) of reaction R_1 (in equation (11)). At the
same time the RNA-f present is rapidly broken down. Since this
RNA moiety is now formed to only a very limited extent, virtual-
Similarly our recent results show:

\[(R_1 + R_3) < (R_2 + R_4)\]  

(16)

Similarly Fig. 6 shows:

\[(R_1 + R_3) \leq (R_2 + R_4)\]  

(20)

Since the breakdown of RNA-f is most likely much greater than the formation by Day 7 after wounding, synthesis of this compound seems to have virtually ceased by Day 11. \((F_1)\) is approaching zero under these circumstances. Reaction \(R_2\) will take care of the remaining RNA-f, i.e.:

\[R_2 > R_1\]  

(21)

However, \(R_3\) is still larger than \(R_4\), although \(R_4\) is probably appreciable because of the relatively high concentration of RNA-s. However, despite \(R_3\) being greater than \(R_4\), it seems probable that

\[(R_2 + R_4) > R_3\]  

(22)

\(^{32}P\) is taken up at a rate similar to that at Day 5 after wounding, but the label lost will be exclusively from the slow
Fig. 7: Incorporation of P^{32} with time into the RNA fraction of regenerating wound tissue at various intervals after wounding. The data are presented as cpm per mg tissue nitrogen. Each point represents average analyses from 5 or 6 animals.

Solid circles: 5 days, halved: 7 days, open: 11 days after wounding.
RNA-s, while breakdown of RNA-f will only result in unlabeled products. Eventually the uptake of radiophosphate will be limited by the loss of the label from the body.

The data on the incorporation of $^{32}P$ per milligram tissue nitrogen in Fig. 7 support the concept of two types of RNA, being formed at different rates and different times in the regenerating process. The most rapid formation of RNA occurs on the 5th day after wounding; before the 48-hour measurement is over, there is already a very appreciable breakdown of RNA-f. On the other hand, the $^{32}P$ is incorporated much more slowly upon administration of the isotope on the 7th day after wounding, which indicates the slower rate of RNA-s formation. This rate of uptake is even lower on the 11th day after wounding, already leveling off at 24 hours after injection of the isotope. This indicates the equilibrium between formation and breakdown of RNA-s as proposed before.

These two different RNA moieties, one being metabolically very active, the other being relatively stable, do not only show differences in metabolism, but also may show differences in their chemical and probably physical properties. The chemical properties will include primarily their base compositions. Here the data in Table II may offer an additional support for the presence of the two types of RNA in regenerating wound tissue. The purine/pyrimidine ratio decreases between the 5th and
the 15th day after wounding. However, the nitrogenous base compositions at Day 5 and Day 8 on one hand, and the base compositions at Day 12 and Day 15 on the other hand show striking similarities. These similarities are duplicated by the RNA types proposed in the previous pages. At Days 5 and 7 after wounding the main type present will be the rapidly formed RNA-f, while later the data indicate the breakdown of RNA-f and virtually exclusive presence of RNA-s. This might be interpreted that the two RNA moieties proposed are represented - very approximately only - by the base ratios in Table II.

It is interesting to note that these changes in the ratio of the nitrogenous bases and the appearance of the two types of RNA also coincide with the period of formation of collagen. This protein begins to be formed in trace amounts at about the 5th day after wounding. Its concentration rises constantly thereafter (26), qualitatively paralleling the appearance of the two RNA moieties. It might be expected that the RNA responsible for the synthesis of a precursor of such a different protein as collagen, would show significant differences in its base ratio from normal RNAs.

Incorporation of \( P^{32} \) into Deoxyribonucleic Acid of Regenerating Wound Tissue

In Fig. 1 the level of DNA increases up to about Day 8 after
Fig. 8: Incorporation of $\text{P}^{32}$ with time into the DNA fraction of regenerating wound tissue at various intervals after wounding. The data are presented as cpm per micromoles DNA-phosphorus. Each point represents average analyses from 5 or 6 animals.

Solid circles: 5 days, halved: 7 days, open: 11 days after wounding.
wounding; the concentration then levels off and finally decreases to some extent. Data in Table I suggest that the incorporation of $P^{32}$ into DNA two hours after injection is most rapid at the seventh day after wounding. Fig. 8 suggests the same picture. In Fig. 9, however, where the activity of $P^{32}$ is calculated on the basis of total tissue nitrogen, the rate of incorporation appears most rapid on the 5th day after wounding, followed by the 7th and then the 11th day. To evaluate this apparent discrepancy between the data in Fig. 8 and Fig. 9, let us assume the following mechanism:

\[
\begin{align*}
\text{"Tissue phosphate pool"} & \xrightarrow{k^n} \text{DNA} \xleftarrow{k^1} \text{nucleotides} \\
\end{align*}
\]

(23)

"Nucleotides" mean the deoxyribonucleotides of the ASP. The ASP in turn is in equilibrium with the phosphate in the rest of the body. "Tissue phosphate pool" represents a possibly fictitious internal accumulation in the wound tissue of monomucleotides and oligo- and poly-deoxyribonucleotides of different sizes which are formed by DNases of the wound tissue. Such DNases have been found in wound tissue shortly after infliction of the wounds (25, 103, 114). DNases of various types have also been found especially in rapidly growing and proliferating tissues (6, 38, 46). These DNases usually produce relatively few mono-nucleotides.

From the mechanism in Equation (23) the following equations may be set up:
Fig. 9: Incorporation of \( P^{32} \) with time into the DNA fraction of regenerating wound tissue at various intervals after wounding. The data are represented as cpm per mg tissue nitrogen. Each point represents average analyses from 5 or 6 animals.

Solid circles: 5 days, halved: 7 days, open: 11 days after wounding.
\[ R' = k'(\text{molecules})(F') \]  
\[ R'' = k''(\text{DNA})(F'') \]  
\[ R''' = k'''(\text{Tissue phosphate pool})(F'''') \]

where \( R' \), \( R'' \), and \( R''' \) are the velocities of the three reactions of the mechanism of equation (23), \( k' \), \( k'' \), and \( k''' \) the appropriate rate constants, \( F' \), \( F'' \), and \( F''' \) the concentrations of the enzymes and other factors involved in the three reactions. From Fig. 1 we can derive the following relations:

At Day 5 after wounding: \((R' + R''') > R'' \)

At Day 7 after wounding: \((R' + R'''') = R'' \)

At Day 11 after wounding: \((R' + R''') \leq R'' \)

Fig. 8 gives the following information:

\[ z_5^R < z_{11}^R < z_7^R \]

where \( z_5^R = R'_5 + R'''_5 - R''^5 \)

the sum of the three reaction velocities at Day 5 after wounding. \( z_7^R \) and \( z_{11}^R \) are the respective sums of reaction velocities on the other two experimental dates.

However, from Fig. 9:

\[ z_5^R > z_7^R > z_{11}^R \]

Since the unlabeled "tissue phosphate pool" would contribute nothing to the labeling of DNA, the results in Fig. 8 would be expected to give a different picture from those presented in Fig. 9. In Fig. 8 the activity is based on the amount of phosphate in the DNA, while in Fig. 9 the activity is based on the
amount of nitrogen in the regenerating wound tissue. Thus, the "tissue phosphate pool" dilutes the radioactivity shown in Fig. Fig. 8, but not that in Fig. 9.

If we compare the situation on the fifth and on the seventh day after wounding as described in Fig. 9, then

\[(R' + R'' - R^*)_5 > (R' + R'' - R^*)_7.\]  

(33)

One may assume that \(R'_5 = R'_7\). This assumption is based on the fact that the \(k^1\) and the concentration of nucleotides (see Fig. 5) are the same on all experimental dates. The terms \(P'_5\) and \(P'_7\) may also be considered to be comparable, since the tissue is proliferating rapidly on both the 5th and on the 7th day after wounding.

It has been shown that the leucocytes invading the wound, even at 48 hours after wounding, are extensively destroyed (25). The DNA from these cells will contribute importantly to the "tissue phosphate pool". However, this contribution becomes less important with time and hence will be expected to be greater on the fifth than on the seventh day after wounding. Therefore

\[R''_5 \gg R''_7.\]  

(34)

Even if \(R''_5\) and \(R''_7\) should be the same (this will probably not be the case) they would be relatively small in both cases. From these considerations, we find that

\[R'5 > R'7\]  

(35)

\[R'5 > R''_5 \gg R''_7\]  

(36)

\[R''5 \simeq R''7 \ll R'5.\]  

(37)
Therefore Equation (33) will hold.

Because of these conclusions the rate at Day 5 after wounding will be higher than on Day 7 (Fig. 9). If one considers specific activities, the rate at Day 5 will appear to be lower than the rate at Day 7 after wounding because of the non-radioactive reaction $R''\uparrow_5$ (Fig. 8).

Comparing the situation at Day 7 and Day 11 after wounding, the problem appears to be much simpler. From Equation (32) it will follow:

$$\frac{x}{7} R > \frac{x}{11} R.$$  \hspace{1cm} (38)

At the 11th day after wounding $R''\uparrow_{11}$ will decrease, since the tissue is not so actively proliferating as in the beginning. $R''$ will be low in both cases. The contribution of $R''$ will be doubtful in both cases; especially on the 11th day after wounding the enzymatic breakdown ($R''\downarrow_{11}$) will be partly substituted by a mechanical removal of cells. At this time the wound tissue assumes an epidermal character (66).

**Amino Acids in Wound Tissue**

Since marked variations were found in the levels and in the metabolism of nucleic acids of regenerating wound tissue, it was thought to be of interest to examine the concentrations of characteristic amino acids in this tissue. The changes in the level of amino acids might be a way of relating nucleic acids
to protein metabolism in this tissue. Dunphy and Udpa (26) had shown that the hydroxyproline concentration increases during wound tissue regeneration. This amino acid exists only in collagen. It is found in this protein in a concentration of approximately 13 percent (9). Tyrosine may be considered to be a characteristic amino acid of "metabolically active proteins", i.e. of proteins which are significantly metabolized and are involved in the metabolism of cells. Tyrosine is found in these proteins in various amounts. Serum albumin contains ca. 4.5 percent tyrosine. Tyrosine is also found in a concentration of about one percent in collagen. Tryptophane is also present in all metabolically active proteins to the extent of about 1.5 percent; it is not found in collagen.

For the determination of tyrosine and tryptophane the method of Folin and Ciocalteau (30) was used, for the analysis for hydroxyproline the modified method of Neumann and Logan (80, 81). The method of Folin and Ciocalteau determines all the tyrosine and about 90 to 95 percent of the tryptophane (88).

The protein residues remaining after the removal of nucleic acids in the previous experiment were hydrolyzed and analyzed for tyrosine plus tryptophane. For the determination of hydroxyproline rats were maintained and wounded as described above. After 5, 7, 12, and 18 days groups of these animals were sacrificed, their wound tissue collected and hydrolyzed in screw cap
Table V

AMINO ACIDS IN REGENERATING WOUND TISSUE

<table>
<thead>
<tr>
<th>Days after wounding</th>
<th>Amino Acids</th>
<th>Protein</th>
<th>Total Protein N</th>
<th>Nitrogen found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HO- proline</td>
<td>Tryp +</td>
<td>m.a.p.* total</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>collagen</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Coll.- m.a.p.* total</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Milligrams per gram Wound Tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>5</th>
<th>7</th>
<th>12</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>3.5</td>
<td>5.5</td>
<td>8.7</td>
<td>14.5</td>
</tr>
<tr>
<td>7</td>
<td>5.5</td>
<td>4.3</td>
<td>4.6</td>
<td>67</td>
</tr>
<tr>
<td>12</td>
<td>8.7</td>
<td>4.6</td>
<td>67</td>
<td>134</td>
</tr>
<tr>
<td>18</td>
<td>14.5</td>
<td>4.7</td>
<td>112</td>
<td>60</td>
</tr>
</tbody>
</table>

* metabolically active proteins
tubes with alkali. Analysis for hydroxyproline and total nitrogen were performed.

Table V shows the results of these experiments. It confirms the previous observations of Dunphy and Udupa (26) that the hydroxyproline concentration and thus the collagen content of wound tissue increases as healing progresses. However, appreciable deposition of this protein begins only after the 8th day after wounding.

The tyrosine plus tryptophane content of the wound tissue protein shows an essentially constant level over the entire period of observation.

The amount of each type of protein may be calculated from the amount of amino acids found, by assuming that collagen contains 13 percent hydroxyproline and 1 percent tyrosine, and that 6 percent tyrosine plus tryptophane are found in the metabolically active proteins. We assume that metabolically active proteins are similar to serum albumin which contains 4.5 percent tyrosine and 1.5 percent tryptophane (9). The protein content is relatively low at the beginning (10.8 percent), where the wound is very "mushy" and highly hydrated, but increases during the healing period to 17.2 percent on the 18th day after wounding. At this time the wound tissue is dry and has epidermal characteristics.

The amount of collagen and metabolically active proteins
in wound tissue may now be recalculated, based upon protein nitrogen and thus compared with the values obtained for total tissue nitrogen. This value for total tissue nitrogen appears to be in good agreement with previous findings of Williamson and Fromm (133). At first the nitrogen present appears to be all protein, within the experimental error. This indicates the presence of leucocytes and fibroblasts which invaded the area after the infliction of the wound. Similarly at the end of the observation period, when the wound is dry and skin-like and little metabolic activity is taking place, 90 percent of the wound tissue nitrogen is protein. At the intermediate periods, the wound shows active metabolism. This might well involve smaller moieties and the relative amount of protein could be expected to be lower. That this is the case can be seen from Table V.
CHAPTER IV

SUMMARY

As indicated in the introduction, there existed virtually no reliable information about nucleic acids in regenerating wound tissue. This dissertation attempted to furnish some basic information on the metabolism of nucleic acids in this tissue.

It was found that at about the eighth day after wounding both RNA and DNA exhibit a peak in concentration in wound tissue on the basis of tissue nitrogen. In the case of DNA this may be more apparent than real. The concentration of DNA seems to remain approximately constant after this date when calculated on the basis of tissue weight.

The metabolism of the nucleic acids exhibits several peculiarities. RNA is apparently formed in two different moieties. These two compounds exhibit different metabolic properties, one being formed and broken down very rapidly, while the other type seems to be synthesised slowly but continually over the observed healing period. The "fast" RNA apparently ceases to be formed at about the eighth day after wounding; from this time on it is virtually only catabolised. These two different types of RNA may eventually be responsible for the formation of different
types of proteins. As is to be expected two completely different RNA moieties will be necessary to facilitate the formation of two so different compounds, like the normal albumin-type plasma proteins and collagen, and more probably its intracellular precursor.

An interesting observation in this direction was made on the base ratios of RNA. During the early stages of the healing period, the purine/pyrimidine ratio is close to unity, while from Day 12 on it drops to about 0.75. This too suggests differences in the properties of RNA. These observations are paralleled by the appearance of collagen in the wound tissue. This fibrous protein begins to be deposited in appreciable amounts after the eighth day after wounding. However, the amount of metabolically active protein seems to remain constant over the entire observed healing period.

DNA shows the most rapid $P^{32}$ incorporation on the seventh day after wounding, when calculated on the basis of phosphorus concentration. On the other hand, when put on the basis of nitrogen, wound tissue collected on the fifth day after wounding seems to exhibit the greatest mitotic activity. This suggests the participation of non-labeled material from some other source than the ASP pool on the fifth day after wounding. Since at the beginning of the healing period, nuclear components from necrotic material are broken down a "tissue phosphate pool" does not
seem to be impossible. DNases produce relatively large amounts of oligo and polynucleotides which do not exchange their phosphorus readily with the equilibrated ASP pool. This "tissue phosphate pool" may play a significant role during the early stages of wound tissue regeneration.

Trauma causes not only changes in the protein metabolism of the whole body, but apparently also in the nucleic acid metabolism of the injured organism. It was found that on the eighth day after wounding RNA is mobilized both from liver and kidney and the ASP fraction of these tissues rises rapidly. It is interesting to note that this mobilization occurs at the time of high nucleotide utilization, when both RNA and DNA are synthesized very rapidly in the regenerating wound tissue. Following this decrease in RNA concentration in the non-regenerating tissues, the RNA level begins to rise again. While RNA levels in liver and kidney fluctuate considerably during the healing period observed, the metabolically stable DNA does not change its amount in these tissues.
ADDENDUM

After completion of this dissertation a publication by Yasuhiro Hosoda was brought to the authors' attention (Keio Journal of Medicine 9: 261 (1960)).

Hosoda's investigations, carried out on male mice duplicate experiments # 1 and # 2 of this dissertation. His findings show a peak level of both DNA and RNA on the eighth day after wounding, as reported in this dissertation. His tracer experiments, however, cannot be compared with the results in this dissertation, since only the P³² uptake 16 hours after injection of the tracer at various times after wounding was reported. The increase in hydroxyproline content of the wound tissue shown in Table V is confirmed by the work of Hosoda. The conclusions in this paper about the possible interrelationships between RNA and the biosynthesis of collagen precursor are similar to those in this dissertation.

To summarize, the findings of Hosoda support and confirm the results and conclusions in this dissertation.
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APPENDIX I

Vitamin Diet Fortification Mixture (Nutritional Biochemicals Co.)

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<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Unit</th>
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</thead>
<tbody>
<tr>
<td>Vitamin A Concentrate</td>
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<td>g/kg</td>
</tr>
<tr>
<td>(200,000 units per gram)</td>
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<td></td>
</tr>
<tr>
<td>Vitamin D Concentrate</td>
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<td>&quot;</td>
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<tr>
<td>(400,000 units per gram)</td>
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<tr>
<td>Alpha Tocopherol</td>
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</tr>
<tr>
<td>Ascorbic Acid</td>
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<td>Inositol</td>
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<tr>
<td>p-Aminobenzoic Acid</td>
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<td>Niacin</td>
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<tr>
<td>Riboflavin</td>
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<td>Pyridoxine Hydrochloride</td>
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<tr>
<td>Thiamine Hydrochloride</td>
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<tr>
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<tr>
<td>Biotin</td>
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<td>Folic Acid</td>
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<tr>
<td>Vitamin B₁₂</td>
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APPENDIX I (continued)

H. M. W. Salt Mixture (Nutritional Biochemicals Corp.)


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<th>Ingredient</th>
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<td>Calcium Carbonate</td>
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</tr>
<tr>
<td>Magnesium Carbonate</td>
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</tr>
<tr>
<td>Magnesium Sulfate (7 H₂O)</td>
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</tr>
<tr>
<td>Sodium Chloride</td>
<td>6.9 %</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>11.2 %</td>
</tr>
<tr>
<td>Potassium Phosphate (monobasic)</td>
<td>21.2 %</td>
</tr>
<tr>
<td>Ferric Phosphate</td>
<td>2.05 %</td>
</tr>
<tr>
<td>Potassium Iodide</td>
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</tr>
<tr>
<td>Manganese Sulfate (H₂O)</td>
<td>0.035 %</td>
</tr>
<tr>
<td>Sodium Fluoride</td>
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</tr>
<tr>
<td>Aluminum Potassium Sulfate</td>
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</tr>
<tr>
<td>Copper Sulfate (5 H₂O)</td>
<td>0.09 %</td>
</tr>
</tbody>
</table>

Alphacel (Nutritional Biochemicals Corp.)

Non-nutitive Cellulose
APPENDIX II

LIST OF ABBREVIATIONS USED

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

AMP  adenine-monophosphate
ATP  adenosine triphosphate
ASP  acid soluble phosphate (phosphorus)
PPI  inorganic pyrophosphate
r    ribose
dr   deoxyribose
DNA  deoxyribonucleic acid
RNA  ribonucleic acid
RNP  ribonucleicprotein
s-RNA soluble ribonucleic acid
aa   amino acid(s)
coll. collagen
m.a.p. metabolically active protein(s)
tyr  tyrosine
tryp tryptophane
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-prol.</td>
<td>hydroxyproline</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloracetic acid</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
</tbody>
</table>
APPROVAL SHEET

The dissertation submitted by Wilhelm Gusahlbauer has been read and approved by five members of the faculty of the Stritch School of Medicine of Loyola University.

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

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

DOCTOR OF PHILOSOPHY.

May 22, 1961

Date

Signature of Adviser