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The Utilization of Sulfur Amino Acids During the Healing of Experimental Wounds

Herbert Jerome Fromm
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THE UTILIZATION OF SULFONAMINE ACIDS
IN THE HEALING OF
EXPERIMENTAL WOUNDS

by

Herbert J. Fromm

Library
Stritch School of Medicine
Loyola University

A Thesis 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

1954

LIFE

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"Inhibition of Urea Formation by Pentobarbital", Arch. Biochem. Biophys., 32, 226 (1951); "Metabolism of Protein Nitrogen During the Healing of Experimental Wounds", Fed. Proc., 10, 270 (1951); "Relation of Protein Nutrition to the Healing of Experimental Wounds", Proc. Soc. Exp. Biol. & Med., 77, 302 (1951); "Effect of Cystine and Methionine on the Healing of Experimental Wounds", Fed. Proc., 11 (1952); "Effect of Cystine and Methionine on the Healing of Experimental Wounds", Proc. Soc. Exp. Biol. & Med., 80, 623 (1952); "Nitrogen and Sulfur Metabolism During the Healing of Experimental Wounds", M. S. Thesis, Biochemistry, Loyola University, Chicago, Illinois, June, 1952; "Distribution of Methionine S^{35} During the Healing of Experimental Wounds", Fed. Proc., 12, 291 (1953); "Utilization of Sulfur Amino Acids During Healing of Experimental Wounds", Proc. Soc. Exp. Biol. & Med., 83, 329 (1953); "Influence of Experimental Wounds on Metabolism of Sulfur", Fed. Proc., 13, 322 (1954)

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CHAPTER I

STATEMENT OF THE PROBLEM

Since ancient times the phenomenon of regeneration has stimulated man's curiosity. Many questions on this subject which were vague and nebulous to our predecessors remain unanswered today. Although a number of empirical facts concerning regeneration have been recorded, no general mechanism to account for it has been presented to date.

Possibly, one of the reasons for this labyrinth is the term regeneration itself. Such diverse phenomena as wound healing, growth, replacement of skin, hair, blood, etc., as well as metabolic turnover, may all be considered to be types of regeneration. There can be no doubt all these subdivisions of regeneration are intimately related. The relationship, however, is confused by differentiation, a term which by definition pertains to macrophenomena. Thus, although skin and blood undergo continuous regeneration, due to the different intracellular composition of the two tissues, there is an inherent difference in the type of regeneration.

Because wound healing, growth, and replacement are closely related, it may be assumed those factors which affect growth will necessarily alter wound healing and replacement. Generally, this has been found to be true. A well nourished animal will grow more rapidly than one suffering from

malnutrition, and, similarly, wounds in the former will heal at a faster rate than those in the starved animal.

For regeneration to occur at all, nutrients must be accessible to the newly developing cell. No distinction can be made in this respect between exogenous and endogenous sources, for both become equivalent once the nutrient has been assimilated.

Of the various groups of dietary constituents, such as proteins, lipids, and carbohydrates, the proteins appear, as will be shown later, to be of paramount importance in the process of wound healing. The proteins and amino acids can not only be utilized for energy purposes, via such mechanisms as deamination and glycolysis, but also supply materials for the synthesis of the most important components of the cell.

The healing wound of course, is limited in its growth and proliferation by substances which these particular wound cells require. Strictly speaking, therefore, it cannot be said that one particular class of nutrients is more important than another.

In the subsequent discussion, particular emphasis will be placed upon the role that certain amino acids play in the healing of experimental wounds. Previous work (Williamson, et al., 1951, 1951a, 1952, 1952a; Fromm, 1952) indicated the sulfur amino acids, methionine and cystine, are capable of accelerating the healing process. These amino acids appear to be unique in this respect with regard to wound healing in that they can elicit this effect even in the absence of dietary protein. The purpose of this thesis will be, primarily, to attempt to establish the role these

substances play in the regeneration of wound tissue.

CHAPTER II

INTRODUCTION

In general, the phenomenon of growth can be looked upon as having two principal aspects. First, it can occur by cell division, and secondly by increased protoplasm formation, i.e., increased numbers and increased volume. In the adult animal, blood corpuscles, hair follicle cells, and epidermal cells grow by cell multiplication. Skeletal muscle and nervous tissue grow by cell enlargement or increased cell protoplasm formation. Both types are observed in the healing wound, as well as in the maturing young animal.

Growth, wound healing, and all the processes of the living system are associated with the intake and utilization of nutrients. Dietary proteins furnish amino acids which are the building blocks of tissue protein. These tissue proteins are the matrix of the living system and are the centers around which the dynamic equilibrium of life develops. There are no protein reserves in the sense of carbohydrate reserves, e.g., glycogen, but the body is capable of utilizing tissue proteins to maintain the integrity of essential nitrogen containing structures. Whipple et al., (1946) and Schoenheimer and Rittenberg (1940) have stressed the dynamic equilibrium associated with proteins and have shown that parts of one type of tissue

-5-

protein contribute to the construction of others. Dietary nitrogen (protein nitrogen) entering into the body rapidly becomes a part of this dynamic equilibrium and hence loses its identity. The primary purpose, then, of dietary protein is to maintain the so-called "metabolic pool" which is in a dynamic state of equilibrium with the tissue proteins of the body. The following is a schematic representation of the interrelation thought to exist between dietary amino acids, tissue proteins, and the metabolic pool of nitrogen (Futon and Simmonds, 1953):

Certain amino acids have been shown to be essential for growth (Rose, 1938). Rose (1938) defines an essential amino acid as one which cannot be produced by the body at a rate necessary for normal growth. In the case of the growing rat these amino acids are:

arginine	methionine
histidine	phenylalanine
isoleucine	threonine
leucine	tryptophane
lysine	valine

An amino acid, though necessary for growth, may not be essential when the animal reaches maturity. Such is the case for arginine in the adult rat (Corley, et al., 1938). Further, it is known that histidine and arginine are not essential amino acids for maintenance of nitrogen balance in man (Rose, et al., 1942, 1943). Thus, the requirement for a particular

amino acid by the animal organism depends upon the physiological state and species of the animal. It should be noted particularly that all of the so-called essential and nonessential amino acids must be present in the organism for life to exist. In this respect then, all amino acids characteristic of the living system are essential.

It has been shown that growth can be maintained on a mixture of synthetic amino acids (Madden et al., 1945; Rose et al., 1943. Subsequently, Cannon and his associates (1947) demonstrated the necessity for supplying the essential amino acids simultaneously. If particular groups of the essential amino acids are fed alternately, rather than simultaneously, there is a diminution of nitrogen retention, and relatively poor growth results.

Nitrogen retention, a measure of growth, is correlated with the physiological state. This concept is probably one of the simplest and best methods for evaluating growth. It is the difference between dietary nitrogen intake and urinary plus fecal nitrogen excretion. Mathematically, it may be expressed as:

$$\text{Nitrogen Balance} = I_N - (U_N + F_N)$$

where " I_N " represents nitrogen intake, " U_N " urinary nitrogen excretion, and " F_N " fecal nitrogen excretion.

It is possible to estimate growth or weight gain from nitrogen balance data alone. Analysis of body protein has shown that it contains approximately 16% nitrogen. Muscle, the largest body protein source, makes up about 20% of the body's weight. The following equation can then be used

to calculate weight increments:

$$\text{Body Weight Change} = \text{Nitrogen Balance} \times 6.25 \times 5$$

Positive nitrogen balance can be maintained on a mixture of the essential amino acids. If one of these amino acids is eliminated from the diet, the animal goes into a negative nitrogen balance, which results in nitrogen and protein loss from the body (Hess, 1938). Certain of the essential amino acids have been shown to spare protein nitrogen and thus assist growth when supplemented to a diet containing protein. Cox and co-workers (1947) showed that when methionine is added to casein hydrolysates and fed to rats, it causes a greater nitrogen retention. A reduction in the excretion of urinary nitrogen when methionine was added to a protein-free diet was observed by Miller (1944), and Allison and his associates (1946). The conservation of nitrogen was attributed to a decreased excretion of urea, indicating a more efficient utilization of the tissue amino acids.

Further, Brush (1947) demonstrated, in the rat, that cystine, and all the essential amino acids except phenylalanine, tryptophane, and valine, exert some nitrogen sparing action, but that methionine is the most efficient in this respect. Allison (1949) believes that since a single amino acid can reduce the excretion of nitrogen, when supplemented to a protein-free diet, this indicates a deficiency of that amino acid in the body. Greater conservation of nitrogen could then occur, when the missing amino acid is included in the diet.

Eckert (1948) and Sadasivan (1950) observed a conservation of

sulfur as well as nitrogen when either methionine or cystine is supplemented to the diet. This conservation was a result of a reduction in the excretion of inorganic sulfate and not of ethereal or organic sulfur. This work indicates that methionine and cystine not only spare protein nitrogen, but, also reduce the degradation of the sulfur amino acids, i.e., less amino acid sulfur is oxidized and excreted as sulfate. This would then cause a greater retention of sulfur amino acids in the body.

Sulfur, like nitrogen, is closely associated with protein metabolism and therefore, growth. Sulfur retention is calculated in a manner similar to nitrogen retention. Methionine, cystine, and cysteine are the sulfur containing amino acids found in dietary protein and animal tissues.

Methionine is believed to serve three primary functions in the body:

1. It can be incorporated into tissue protein per se, i.e., it is required for efficient protein synthesis.
2. It can give up its "labile methyl group" to the so-called "methyl pool" and act as a methyl donor (du Vigneaud, 1939, 1940).
3. It can give rise to cystine and cysteine.

Methionine has been shown to be the only sulfur amino acid essential for growth (Rose, 1938). It can also help meet the body's requirement for cystine and cysteine by supplying the sulfur atom (Rose, 1941; Tarver, 1941, 1942). A mechanism for the conversion of methionine to cystine and cysteine was suggested by du Vigneaud (1944) and Binkley (1951). This conversion, which requires the participation of the ATP system (Binkley, 1944) and pyridoxal phosphate as a co-enzyme factor (Binkley, 1950, 1952) is represented as follows:

Methionine

Homocysteine

Homocysteine

Serine

Cystathionine

Cysteine

Homoserine

Cysteine

Cystine

It should be noted particularly, that the formation of methionine from cystathionine does not occur in the rat, i.e., the overall reaction is not reversible.

Cystine facilitates growth only when methionine is present in amounts insufficient to meet the total sulfur amino acid requirement of the diet (Almqvist, 1945; Kratzer, 1948; Womack, 1951). At best, cystine can replace one-sixth of the body's methionine requirement. Thus, cystine is always capable of exerting some methionine sparing effect, but the nonessential amino acid cannot fully compensate for a methionine deficiency below the minimum requirement. Rat diets containing 18% casein as the sole source of protein are slightly deficient in methionine and may be improved for growth by adding either cystine or methionine (Malford, 1942; Pavcek, 1941).

Inorganic sulfate can be incorporated into cysteine in the presence of the rat liver enzyme, cysteine desulfurase (Fromageot, 1939). The reaction, which is reversible, is believed to proceed in the following manner:

This mechanism might explain the conversion of cystine and cysteine to carbohydrate.

Inorganic sulfate is recognized to be the end-product of amine acid sulfur catabolism. Because methionine sulfur is oxidized more slowly to sulfate than is cystine sulfur (Lewis, 1936), it is thought that the conversion of methionine to cystine is a prerequisite for the oxidation of methionine sulfur to sulfate.

Cysteic acid has been postulated as an intermediary in the breakdown of cysteine to taurine. When the former amino acid was fed to starved dogs there was an increased formation of taurine as indicated by a rise in the excretion of taurocholic acid in the bile (Virtue, et al., 1938). An enzyme present in the liver (Blaschko, 1942) and spleen (Medes, et al., 1942) has been demonstrated to be capable of decarboxylating cysteic acid to taurine.

Medes (1939) has reported that certain liver enzymes have the ability to oxidize cysteine and cystine sulfur to sulfinic acid, which can

then be converted to sulfate. The intermediary steps in the conversion of cysteine to sulfate and taurine have been elucidated, and the pathway is shown in the following schematic representation (Awapara, 1953, 1953a; Chatagner, et al., 1951):

Recent experimentation with eviscerated dogs has added additional evidence to the belief that the liver is the site of amino acid sulfur oxidation (Forkner, et al., 1951).

Under normal conditions, approximately 80% of the total sulfur in the urine exists as sulfate. Starvation results in a decreased excretion of sulfate. The residual sulfur is found as neutral sulfur (methionine, cystine, and cysteine) and ethereal sulfates (sulfuric acid esters).

Both cystine and cysteine are known to exist in most proteins. The tripeptide glutathione (γ-glutamylcysteinylglycine) functions in oxidation-reduction reactions and plays a role in maintaining the sulfhydryl enzymes. Keratins of the hair and skin as well as the hormone insulin contain appreciable amounts of cystine. It was previously thought that the sulfhydryl groups of the Malpighian layer of epidermis are converted to disulfide of keratin (Walker, 1924; Giroud, et al., 1951). Recent

investigation, however, indicates that the disulfide content of the Malpighian layer of skin is in the same range as that of the horny or outer layers. These findings support the theory that epidermal keratinization begins in the Malpighian layer of this tissue (Carruthers, et al., 1953, Mascon, et al., 1952; Van Scott, et al., 1954).

Ethionine, a homolog of methionine, has been demonstrated to be toxic to the animal organism (Goldberg, et al., 1950). It is thought that ethionine acts as a competitive inhibitor in both the conversion of methionine to cystine and the incorporation of methionine into proteins (Simpson, et al., 1950a). These effects can be reversed by the addition of methionine to the diet.

Radioactive isotopes have been used successfully in recent years as a means of studying protein synthesis, both in vivo and in vitro. The synthesis of proteins is considered to be a slow reaction whether it is measured as a net gain of protein in a young growing animal, or as turnover, where the amount of protein remains the same (Berzock, 1950). Schoenheimer and co-workers (1939) estimated that the half-life of liver protein is about seven days in the adult rat. Other workers have reported differential rates of uptake and amino acid release by different tissues. In Table I is shown data on the relative rates of incorporation of S^{35} labeled methionine into organ and tissue proteins twenty-four hours after the feeding and injection of methionine- S^{35} to rats.

An insight into the mechanism of protein synthesis has been obtained using radioactive materials. Melchior et al. (1948) found that

Table I

Relative Rates of Methionine Incorporation Into
Tissue Proteins After Administration
of ^{35}S Labeled DL-Methionine

	Plasma = 100	
Liver	80a	77b
Kidney	95a	114b
G.I. Tract	157a	154b
Spleen	57a	
Brain	21a	23b
Testes		33b
Heart		27b
Muscle		19b
Carcass	15a	

a - Uptake 24 hours after feeding methionine (Tarver, et al., 1948)

b - Uptake 6 hours after I.V. injection of methionine (Friedberg, et al., 1948)

non-proliferating E. coli incorporated s^{35} labeled methionine into their proteins. Mechanical damage to the cell, such as results from homogenisation, decreases the rate of uptake of amino acids by proteins, i.e., cell integrity is required for efficient protein turnover and synthesis (Melchior, et al., 1947; Winnick, et al., 1948).

Peptide synthesis under physiological conditions is not merely a reversal of hydrolysis. It must be coupled with an energy donating reaction (Borsook, et al., 1935). Anaerobiosis and inhibitors of oxidation and phosphorylation decrease the uptake of labeled amino acids into proteins (Borsook, et al., 1950). Conditions which limit the release or utilization of energy (anaerobiosis, cyanide, 2,4 dinitrophenol) have been found to depress the liberation of labeled amino acids from proteins in turnover studies (Simpson, 1953).

Feeding experiments have shown that an essential amino acid is ineffective for growth or maintenance unless all the other indispensable amino acids are fed simultaneously (Cannon, et al., 1947; Geiger, 1947, 1948). This indicates that all the amino acids must be available for protein synthesis to occur. On the other hand, in vitro studies demonstrate that the uptake of a single amino acid into proteins is independent of that of other amino acids (Borsook, et al., 1950; Simpson, et al., 1950). It becomes apparent then that turnover (incorporation of an amino acid into protein and its release) and net protein synthesis are distinctly different entities. Undoubtedly, both processes occur in the living organism, and as yet are indistinguishable. When an amino acid is incorporated into a

protein two peptide bonds are broken and two are resynthesized. Protein synthesis requires the formation of peptides from amino acids.

Tarver (1950) has shown that many factors will influence the incorporation of a labeled amino acid into protein. Among these are: size of administered dose, and the nutritional state of the animal. It is possible to measure protein synthesis and turnover together after the administration of a labeled amino acid. Tarver (1950; Tarver et al., 1948) refers to these two factors as replacement. This concept appears to be valid provided that no attempt is made to differentiate turnover from synthesis.

It is possible to estimate the fraction of a labeled amino acid which is replaced in a given tissue at two intervals of time after the administration of the labeled amino acid. Calculations are based upon specific activity (ratio of labeled atoms to total number of isotopic atoms). By plotting this factor against time after the administration of the labeled amino acid, it is possible to calculate average protein half-lives. The average protein half-life is suggestive of turnover, protein lability, protein synthesis, and protein metabolic activity.

Synthesis and protein turnover appear to be dependant upon the protein and free amino acid content of the diet. High protein rations cause decreased labeling (due to dilution), while the converse is true when animals are fed a low protein diet. Dietary methionine appears to favor the retention of S^{35} labeled methionine (Tarver, et al., 1952).

CHAPTER III

WOUND HEALING AND PROTEIN METABOLISM

The healing of the clean incised wound may be divided into three main phases (Locallio, et al., 1943):

1. The phase of traumatic inflammation, in which hyperaemia and exudation occur;
2. The phase of destruction, in which the wound is freed of dead and dying cellular elements. These two periods constitute the "lag phase" of wound healing, during which time healing progresses slowly.
3. The phase of proliferation, in which fibrous-tissue formation, i.e., fibroplasia, contraction, and epidermization occur. The secretion of collagen in wound tissue takes place during this phase.

Although Clark in 1919 reported that a high protein diet increased the rate of skin closure in the skin of the wounded dog (Clark, 1919), no relationship was known to exist at that time concerning the healing of wounds and protein metabolism. In recent years, much work has been undertaken in an attempt to accelerate the healing of wounds. For the most part, however, little progress has arisen from these efforts.

Harvey and co-workers (1930) have shown that although the feeding of a high protein diet did not influence the lag period in the healing

wound, it did accelerate fibroplasia. Other reports which are in agreement with this work indicate, however, that the level of dietary protein does affect the lag period of wound healing to some extent (Chalkey, et al., 1945; Co Tai, 1945; Green, et al., 1953; Kobak, et al., 1947; Localio, et al., 1943, 1948; Maconshi, et al., 1949; Mariani, et al., 1950; Morris, et al., 1945; Sandbloom, et al., 1953; Thompson, et al., 1938; Williamson, et al., 1951, 1951a). Undoubtedly, the measurement of wound healing employed by Harvey and his associates was not sensitive enough to determine differences in the healing rate during the lag phase, at which time wound strength is at a minimum.

From the foregoing discussion on the phases of wound healing, it might appear that proliferation and the lag period do not overlap, i.e., no collagen is deposited in the wound tissue during the lag period, and thus no wound strength can be observed. That overlapping does take place becomes apparent when it is realized that lag period measurements entail wound strength determinations. The latter measurement, a result of protein accretion, can occur only during the phase of proliferation.

When wound healing is considered as being essentially a growth process, the question arises as to whether the healing of wounds takes precedence over normal body growth and maintenance. Howes and co-workers (1933) report that complete starvation does not alter the shape of the healing curve of wounds in the adult rat. On the other hand, starvation retards fibroplasia and final healing in the wounds of growing rats. This would suggest that the healing of wounds is of lesser importance to the

organism than so-called normal body growth. This conclusion would probably never have been presented had these workers considered differences in body weight. In the adult animal, a greater "protein pool" is available to the healing wound than in the lighter, growing animal. On a starvation diet, the tissues become the only source of amino acids for wound protein synthesis. It seems logical, then, in the light of this consideration, that the wounds in the heavier animals should display more rapid healing rates. It is imperative, therefore, that in any investigation dealing with the healing of wounds, all animals should be of the same body weight.

Hypoproteinaemia, a result of starvation, is thought to delay fibroplasia in wounds by first interfering with the osmotic relationship between plasma and tissue. This would then decrease the amount of protein that could be withdrawn from the plasma for use by the tissue for repair (Localio, et al., 1948; Mariani, et al., 1950; Rhoads, et al., 1942; Thompson, et al., 1938).

Recently, investigators have tested many essential amino acids in the hope of finding some that are capable of accelerating the healing rate of wounds. Lysine, tryptophane, and valine were found to have no significant effect on the healing rate of wounds in injured rats when supplemented to low, medium, or high protein diets (Morris, et al., 1945). These workers also observed a decreased healing rate of wounds in injured animals fed a high protein diet. This would suggest poor utilisation of proteins for healing purposes when dietary carbohydrate is at a low level.

Williamson (1951a) has shown that histidine is incapable of increasing the healing rate of wounds in rats fed casein diets. However,

the administration of 1% DL-methionine to the diet of experimentally burned animals was demonstrated to speed up markedly the healing rate of burns (Croft, et al., 1945, 1945a; Peters, 1945). Parenteral administration of DL-methionine produced similar effects in the wounds of protein depleted experimentally injured rats (Localio, et al., 1948). This was subsequently confirmed by histological studies (Perez-Tamayo, et al., 1953).

When cysteine was tested to determine its influence on the healing wound, it was reported that this sulfur amino acid was unlike methionine in that it had no observable effect on the healing process (Croft et al., 1945, 1945a). This work suggests that the injured organism requires methionine per se. Another possibility is that methionine might act as a methyl donor in the healing of wounds. Further investigation in the effect of the sulfur amine acids on the healing of wounds was apparently halted by the publication of these papers.

In 1929 Cuthbertson reported that after experimentally induced fractures, animals displayed an increased urinary nitrogen loss, and that this material is not derived merely from the area of injury. This situation could not be altered by the feeding of high caloric or high protein diets. In fact, it has been shown that the greater the proportion of protein in the diet before and after trauma, the greater the post-injury urinary nitrogen loss (Cuthbertson, 1946).

Protein depletion which follows moderate to severe injury is thought to be due to one or more of five main causes (Cuthbertson, 1946).

1. Loss of actual tissue.

2. Loss of blood or exudate from the damaged area.
3. Loss due to infection if present.
4. Disuse atrophy.
5. Loss due to excessive protein catabolism.

It is now believed that this last factor, excessive protein catabolism, is primarily responsible for protein depletion following trauma (Cuthbertson, 1946).

A marked fall in serum protein after burns, suggests that this condition is an indication that serious depletion of the plasma reserves has taken place (Cuthbertson, 1946). It was also reported that the excessive amounts of nitrogen as urea, sulfur as inorganic sulfate, and phosphorus as inorganic phosphate, which appear in the urine after injury are derived from protein, and that the magnitude of the nitrogen to sulfur ratio suggests that they come from muscle protein.

The possible source of protein for the excessive urinary nitrogen loss after injury was speculated upon by Cuthbertson, McGirr, and Robertson (1939). They reported that a more negative nitrogen balance was absent after injury in experimentally wounded animals which had been fed a protein-free diet and whose nitrogen excretion was constant. They suggested that the nitrogen arises from "storage" protein and not essential tissue substances. The term storage protein is supplied by Whipple (1946) who classifies body protein into three main groups:

1. "Indispensable" cell protein, without which cellular life ceases.

2. "Dispensable" protein which the cell can give up in situations such as starvation to supply energy.

3. "Labile" protein which can move in and out of the cell if the plasma protein concentration rises or falls.

Dispensable and labile proteins make up the so-called storage protein.

Until the work of Williamson and his associates (1952, 1952a) was reported, methionine appeared to be the only amino acid capable of decreasing the magnitude of the negative nitrogen balance which characteristically follows injury (Croft, et al., 1945, 1945a, Peters, 1945). Williamson and his co-workers (1951, 1951a) have shown that the rate of wound healing in rats is correlated with the retention of amino acid sulfur in these animals. On the other hand, no relationship was observed between the healing rate of wounds and nitrogen intake or retention. From these experiments, it was concluded that previous reports which dealt with the effect of the level of protein in the diet on the rate of wound healing were indicative of the effect of the level of protein sulfur intake, rather than the effect of the protein itself. This is apparent from the fact that other workers tested the influence of different levels of the same protein on the healing rate of wounds; in which case, the sulfur content of the diet varied directly with the amount of dietary protein.

Balance studies conducted on experimentally injured animals indicated that a larger proportion of sulfur to nitrogen is required during healing than for normal tissue synthesis. This is supported by the following:

1. After injury a positive sulfur balance is observed even when there is a negative nitrogen balance.

2. Increasing the ratio of sulfur to nitrogen by dietary means (by the use of methionine) increases the rate of healing.

Further, these workers observed that there was an amount of protein sulfur, termed excess sulfur, which was retained in excess after injury, to that amount necessary to form normal tissue protein with the retained nitrogen (Williamson, et al., 1951). It was observed that this excess sulfur was correlated with the healing rate of wounds in rats.

Although Croft and Peters (1945, 1945a) found that cysteine was not effective in accelerating the healing rate of wounds in injured animals, Williamson et al. (1952, 1952a) demonstrated that cystine was capable of increasing the rate of wound healing in rats to the same extent as methionine, when the two amino acids were supplemented to the diet of wounded animals in equivalent amounts. These conflicting results suggest that only the oxidized form of the non-essential amino acid is required by the injured animal. It seems doubtful, however, that cysteine which is easily oxidized to cystine in vivo would be without effect in this respect.

Because cystine does facilitate healing in injured animals to the same extent as methionine, per equivalent of diet, it was suggested that the beneficial effects of the latter amino acid on the healing wound could not be due to its methylating property (Fromm, 1952). It was suggested that cystine might be the limiting sulfur amino acid during the healing of wounds, because methionine is converted to cystine in vivo, whereas the

reverse reaction does not occur to any appreciable extent (Williamson, et al., 1952, 1952a). The effects of methionine and cystine on the repair process were found to be similar both in the presence and absence of dietary protein. It seems logical to suppose, that once the requirements for the sulfur amino acids are met by the injured animal, another amino acid might become the limiting factor.

Even though the sulfur amino acids were found to be effective in accelerating the healing rate of wounds in injured rats, the function of these substances has not been ascertained. Layton (1950) and Sylven (1951) have observed sulfate fixation in wound tissue. Would inorganic sulfur, however, account for the relatively high values of excess sulfur found in wounded rats? Wound proteins might possibly require appreciable amounts of the sulfur amino acids. This would then account for the increased healing rates of wounds observed upon the addition of methionine and cystine to the diets of injured rats. On the other hand, the sulfur amino acids might in large part be required by proteins other than those found in wound tissue.

Although the level of protein in wound tissue increases with time and approaches the value for corresponding unwounded tissue (Paul, et. al., 1945), it will be shown that wound proteins contain large quantities of methionine and cystine, and particularly the latter amino acid.

CHAPTER IV

METHODS OF DETERMINING THE HEALING RATE OF WOUNDS

In order to estimate the healing rate of wounds, it is necessary to have some criterion of measurement. At the present time, three methods, other than histological, are employed to evaluate the so-called healing rate of wounds. These methods involve wound potential, wound closure, and wound strength determinations. It will be shown that although all these three procedures are theoretically valid, the wound strength criterion is probably the most practical.

Wound Strength Determinations

A relatively simple and accurate method for measuring healing is the wound strength or rupture point measurement. The instrument itself is easily assembled, and the results obtained fairly reproducible.

Wound strength has been shown to be a valid measure of wound healing (Harvey, et al., 1930; Howes, et al., 1933; Kobak, et al., 1947). In fact, most of the workers in the field have adopted some modification of this technique. Although wound strength is not an absolute measure of the healing of wounds, it is correlated with histological findings on the number of wound tissue fibroblasts (Kobak, et al., 1947).

Charney, Williamson, and Bernhard (1947) described an apparatus for measuring the strength of wound tissue which minimized subjective error. Standard samples of a clean incised wound are allowed to heal. At a definite time interval after wounding, the wound tissue is removed, and the strength of the regenerating wound tissue determined. The details of the procedure have been described previously (Fromm, 1952).

When the strength of the healing wound tissue in grams is plotted against time in days, a sigmoid growth curve similar to that shown in Fig. 1 is obtained (Harvey, et al., 1930; Howes, et al., 1933; Kobak, et al., 1947). It can be seen that immediately after injury, wound healing progresses slowly. This period of time constitutes the lag phase of the healing process, and is shown on the curve in Fig. 1 from the origin to point A. Segment A-B on the "S" shaped curve represents maximum wound strength increase with time and corresponds to the protein accretion or proliferation phase of wound healing. Beyond point B, the healing rate of wounds approaches zero, the wound strength having reached a maximum.

It has been shown that the protein accretion phase of regeneration has its onset at about the fifth day after injury and terminates at about the twenty-second day after wounding in the adequately fed animal (Paul, et al., 1948). For practical purposes then, the protein accretion phase of healing is represented graphically in Fig. 1 by segment A-B. It can be seen that this part of the healing curve (segment A-B) is essentially linear. The equation for this line can be expressed as:

$$W = K(t) + c$$

Fig. 1

where "W" is the wound strength per unit section of healing wound tissue, "K" the slope of the line, "t" the time in days after wounding, and "e" the deviation of the curve from the origin.

The average slope, K, can be determined if two or more points are known, using the following equation:

$$\text{Slope(K)} = \frac{W_2 - W_1}{t_2 - t_1}$$

It can be seen that the dimensions of the slope, grams per unit time, is a rate term. The value of the slope(K) is referred to as the healing index, and can be looked upon as being a semiquantitative measure of the healing rate of wounds.

Wound Potential Measurements:

Physiologists have demonstrated that injured and non-injured tissues display a difference in electrical potential (Nahum, et al., 1943). This phenomenon is referred to as the potential of injury. When a tissue is injured repolarisation can no longer take place, and an injury potential is set up between the injured region and the adjacent normal areas. Directly after injury, the potential difference is found to be highly positive. As healing progresses, there is a decrease in the current of injury which is directly proportional to the healing rate of wounds. Barnes and co-workers (1946, 1945, 1951, 1951a, 1952, 1953) have made use of these alterations in electrical potential after injury as a criterion of healing.

The procedure involves touching the wound with an agar salt bridge

dipped into a cup of saline solution. The saline solution is connected by a salt bridge to another cup of saline into which dip chlorinated silver electrodes. These electrodes are attached to a single point Leads and Northrup recording Micromax potentiometer. The potentials are recorded at various intervals after injury. Healing is assessed by plotting wound potential against time.

From a theoretical point of view wound potential measurements are highly acceptable. It has been found, however, that too many extraneous factors limit the practicality of this method. Among these factors are:

1. Excitement of subjects causes wide variations in electrical potential.
2. The experimental animal must be anesthetized. The procedure would therefore be of limited value in metabolism studies due to metabolic alterations induced by anesthesia.
3. Rigorous control of external temperature is needed to prevent fluctuations in wound potential.
4. In most cases precision is poor. Large wounds are necessary for reproducibility.

When the above-mentioned difficulties are understood and circumvented, wound potential determinations may prove to be extremely valuable.

Wound Closure Measurements:

It has been shown that wound closure is correlated with the healing rate of wounds (Morris, et al., 1945; Paul, et al., 1948). The procedure involves the infliction of a circular wound in the skin of the

experimental animal. The wound is held in place with metal disks sutured into the skin. Tissue invagination occurs from the border of the wound, with the ultimate closure of the entire wound. The size of the healing wound is determined at regular intervals. When the reciprocal of this quantity is plotted against time, a graph similar to that shown in Fig. 1 results. This method gives results which are confirmed by wound strength determinations and histological findings.

Like the wound potential method, one of the great advantages of this determination is that the same animal can be used throughout the entire experiment. On the other hand, sutures, disks, anesthesia at the time of measurement, and excessive handling, all necessary in the wound closure method, tend to affect the measurements as well as the wounded animal. Undoubtedly, the greatest deterrent in this procedure is the extremely high incidence of infections in open wounds. Because of these limitations, the wound closure method of determining the rate of healing of wounds is not frequently used.

CHAPTER V

THE EFFECT OF NON-PROTEIN FACTORS ON WOUND HEALING

In the foregoing discussion relating wound healing and protein metabolism, it was stated that in most cases, those factors which affect growth will necessarily influence regeneration, at least to some degree. Any nutrient required by the regenerating tissue must be available to that tissue in order for healing to occur. The nutritional requirement of each cell is determined by its intracellular components.

The synthetic or anabolic reactions which occur during both growth and wound healing require an energy source. It seems logical to assume, then, that the carbohydrates and lipids would necessarily play an important role in this respect. Not only are they capable of "sparing" amino acids for the synthesis of wound tissue, but the oxidation of fats and carbohydrates liberates energy which can be harnessed by the enzymes of the body and utilized for anabolic syntheses.

To support this view, Rossiter and Clark (1944) demonstrated a systemic rise in blood lactic acid, correlated with hyperglycemia, at the ultimate expense of muscle glycogen, in the healing of mammalian wounds. Further, the increase in the level of blood glucose parallels protein mobilization. This work strongly suggests that carbohydrate is a major

energy source for wound protein synthesis.

Certain cell constituents in healing wound tissue have been shown to contain carbohydrate material (Bradfield, 1951). The concentration of chondroitin and mucicetin sulfuric acid esters in both epidermal and granulation tissue increases during the healing of wounds (Sylvén, 1941).

Very little is known concerning the effect of the lipids on the healing of wounds. Fat droplets and glycogen apparently herald the end of proliferation in tissue cultures (Fisher, 1946). This work, of course, cannot be interpreted to mean that regeneration is inhibited by lipid material. However, Whipple (1940) concluded that fats actually retard wound healing. On the other hand, it has been reported that during liver regeneration, following protein starvation, the optimum fat intake for nitrogen balance was as high as 30% of the total food intake (Rogers, et al., 1950).

The adrenal cortical steroids, which are considered to be lipids, appear to promote protein synthesis in liver regeneration (Friedgood, et al., 1950). It has been postulated that the adrenal cortex produces two hormones which are present in the urine in increased amounts in the first twenty-four hours after injury. One, the "N" hormone, is a testosterone like substance which causes a retention of protein and facilitates regeneration. The other, termed the "S" hormone, a cortisone like substance, increases gluconeogenesis and inhibits the repair process. After trauma, the adrenal cortex first secretes an excess of both hormones which appear in the urine. In the later stages of trauma, the "S" hormone appears in

excess. The secretion of the glucocorticogenic steroid parallels the period of nitrogen loss from the body (Cope, et al., 1943). Evidence has also been presented which indicates that cortisone inhibits fibroplasia (Baker, et al., 1950; Blunt, et al., 1950; Howes, et al., 1950; Robson, et al., 1950; Spain, et al., 1950, 1950a; Tauberhaus, et al., 1950). On the other hand, desoxycorticosterone acetate accelerates wound healing in injured animals by promoting fibroplasia (Pirani, et al., 1951).

Ovarian hormones appear to be more effective in promoting wound healing in the female, while testicular extracts are apparently beneficial to the male of the species. This suggests that these steroids function through the specific metabolism of the respective sexes. Some workers have failed to detect an accelerated rate of wound healing in wounded animals given gonadal steroid hormones (Emery, 1937; Davidson, 1945) or adrenal steroids (Noble, 1950). These apparent contradictions concerning the effect of the steroid hormones on regeneration might be explained by the fact that these substances are specific in their action to a particular sex and tissue. Further, varying dose levels undoubtedly influence the organism in a spectrum of ways. This conclusion is corroborated by Williamson and Neumann (1954) who have shown moderate doses of growth hormone promote wound tissue regeneration in rats, while the converse is true at higher dose levels of the hormones.

It was recognized as long ago as 1772 by Lind, that scurvy inhibits the healing of wounds. This finding has been substantiated by a number of workers (Bourne, 1944; Grandon, et al., 1940; Peters, 1948;

Reid, 1948; Penney, et al., 1949) in recent years, who concluded that ascorbic acid is required for the production of an intercellular substance which holds cells together. It has been suggested that this intercellular material is collagen (Hunt, 1941).

The fat-soluble vitamins, A, D, E, and K have not been shown to play any specific role in regeneration. However, vitamin K probably facilitates repair due to its association with the clotting mechanism.

As integral parts of co-enzymes, the B vitamins must be essential for repair. Bosse and his co-workers (1948) demonstrated that biotin, pyridoxine (co-transaminase), and riboflavin (yellow enzyme co-factor) promote healing when fed to injured rats. Pantothenic acid (co-A) (Petrina, 1951) and p-amine benzoic acid (Gucinatta, 1948) when applied to the injured area, accelerate the healing of wounds. Vitamin B₁₂ increases the regeneration of liver proteins after fasting (Sahasrabudhe, et al., 1951). This effect might be due to vitamin B₁₂'s ability to spare methionine for the repair process.

CHAPTER VI

EXPERIMENTAL PROCEDURE AND METHODS

A review of the literature concerning protein metabolism and the healing of wounds suggests the indispensability of the sulfur containing amino acids. That methionine and cystine accelerate repair of wounds in injured animals has been demonstrated. The mode of action of these substances, however, is not known. Undoubtedly, a deficiency of the sulfur amino acids or their metabolic products exists in the injured animal during the healing process. The experimental procedure and methods to be described in this thesis were employed in an attempt to ascertain the function of methionine and cystine during the healing of experimental wounds.

The Experimental Animal

The female albino rat of the Sprague-Dawley strain was the animal used in all experiments. The rat was chosen as the experimental animal since it is easy to handle and takes up little space. Further, its wounds are quite resistant to infection. In order to rule out the effect of the sex hormones, it was important that only one sex be used in these studies on wound healing.

Before any experiment was carried out, the animals were inspected for sniffles, diarrhea, and hematin deposits around the eyes. If these

disease symptoms were present, the animals were discarded and the experiment discontinued.

Among those factors which influence the healing of wounds is body weight. It was necessary, therefore, that the animals in any experiment be of similar weight. The rats employed in these studies weighed 200 ± 20 g. At this body weight the female rat is believed to have reached maturity, and consequently, is not greatly influenced by growth or senility factors.

The rats were housed in metabolism cages and were weighed at regular intervals throughout each experiment.

Diets

The importance of nutrition in the healing of wounds was stressed in the introduction of this thesis. Without an adequate supply of nutrients, all types of growth, including the healing of wounds ultimately cease. By the use of synthetic diets, it is possible to regulate the protein nitrogen and sulfur intake. These data, along with a knowledge of nitrogen and sulfur excretion, allows for the computation of retention (balance) values.

The diet itself permits the study of the effect of various dietary substances on the healing of wounds. Thus, in the absence of dietary protein, it is possible to determine the influence of a single amino acid on the regeneration process.

In the experiments to be described, the animals were placed on a synthetic basal diet for approximately five days. This period of time is referred to as the acclimation period, during which time the rats became accustomed to the synthetic diet and environmental conditions.

In Table II is shown the constituents found in the basal diet. This ration contains all the dietary substances now known to be necessary for the maintenance of the rat. The basal diet, however, is acknowledged to be low in protein content. This protein level (6% casein) was chosen so that the animals would exhibit only slightly positive or negative nitrogen and sulfur balances, i.e., remain in balance.

In some of the experiments to be described, rations containing no protein were used in place of the basal diet. To accomplish this, the basal ration was altered to contain 83% sucrose. This 0% protein ration was employed in studies where S^{35} labeled amino acids were administered to the rats, in which case it was desired to minimize dilution effects. The diets used in the experiments were isocaloric, and thus the possibility of caloric content influencing metabolism or the healing of wounds was eliminated. In cases where amino acids were supplemented to the diet, they were presented on a chemically equivalent basis.

All diets were kept in a refrigerator at a temperature of 5° C. This prevented the lipids from becoming rancid and the food unpalatable.

The food offered the animals was weighed on a triple beam balance, and was in such an amount as to insure total consumption, usually eight grams per rat per day. The diet was placed in feeding cups specially designed to prevent spillage, and so a relatively accurate estimate of food intake could be made. The animals were fed every twenty-four hours, and distilled water was given "ad libitum".

Table II

Composition of Basal Diet

<u>Dietary Substance</u>	<u>gm/ 100 gm Diet</u>
Casein (13.7% N., 2/3% S.)	6
Lard	10
Corn Oil	2
Sucrose	77
Salt Mixture (Hubbel, et al., 1937)	5
<u>Vitamin Supplement</u>	
Thiamine Hydrochloride	1
Riboflavin	1
Pyridoxine Hydrochloride	1
Calcium Pantothenate	4
Inositol	5
p-Amino Benzoic Acid	5
Nicotinic Acid Amide	15
2-Methyl Naphthaquinone	0.5
Choline Chloride	25
Vitamin A (from oleum percomorphum)	1500 IU
Vitamin D (from oleum percomorphum)	210 IU

Wounding Procedure and Wound Strength Determinations

After the acclimation period, the rats were anesthetized with sodium nembutal by intraperitoneal injection (3.5 mg./100 gm. rat). The hair in the region of the back of the neck and shoulder blades was carefully clipped down to the skin with a scissors. In cases where skin wounds were accidentally inflicted, the animals were discarded. A standard four cm. slit wound was made in the skin down to the muscle, in the mid-dorsal region, with a clean, sharp scissors. This area was chosen since it is not easily disturbed by the animal. Wounds located in other parts of the body are often disrupted by the rat, and as a result, uneven healing occurs.

The edges of the clean incised wound were opposed and held in place with four evenly spaced wound clips (11 cm.). Wound clips were used to hold the edges of the wound in place and to insure standard and rapid wound closure.

After a four day interval, the rats were anesthetized lightly with ethyl ether, and the wound clips carefully removed. Care must be exercised at this point to prevent wound disruption. The wounds were inspected daily for signs of infection. However, no cases of infection were noted.

In Fig. 2 is shown the instrument used to determine the strength of healing wound tissue. In the experiments to be described the apparatus was used in the following manner:

Fig. 2

At approximately weekly intervals one-third of the rats were anesthetized with sodium nembutal and four 0.5 cm. sections of healing wound tissue excised. The animals were then sacrificed, and the strength of the standard wound section determined.

A standard sized piece of wound tissue was placed between clamps (1) and (2) of the apparatus shown in Fig. 2. Carbon dioxide under pressure was forced at a standard rate into a mercury reservoir (3) and the mercury transported to a plastic container (4) supported by the wound tissue. An increasing stress was placed upon the wound tissue, which eventually ruptured. The release of a stop-cock (5) caused by the fall of the mercury containing plastic container, opened the otherwise closed system. This automatically stopped the flow of mercury into the plastic container. The plastic container was weighed, and the weight in grams necessary to rupture the standard section of healing wound tissue recorded as wound strength.

Possibly the only limitation of the described instrument is its lack of sensitivity in measuring the strength of wound tissue during the first few days of healing. This was due to the weight, about 100 grams, of the empty container which received the mercury. In order to obtain valid results, no measurements were made until the end of the lag phase, about one week.

Wounding Procedure for Amino Acid Assay Determinations

The work of Williamson and his co-workers demonstrated (1951,

1951a, 1952, 1952a) the retention of methionine and cystine sulfur in the rat after injury. Establishment of the major site of deposition of these amino acids might possibly elucidate the function of these substances during the process of regeneration. For this reason, samples of wound, skin, muscle, and liver tissue were removed from rats convalescing from injury. It became necessary, therefore, to alter the wounding procedure described for wound strength, so that enough regenerating tissue could be obtained for the determination of wound methionine, cystine, and nitrogen.

The animals were treated similarly to those used in the determination of wound strength. After removal of hair from the potential wound area, the outline of a coin (four cm. in diameter) was traced in ink on the skin. A clean, sharp, curved scissors was used to excise the skin down to the muscle over the area of the ink outline. The wound was then allowed to heal. Very few cases of wound infection were noted throughout the studies to be described. In cases where infections were observed, the rats were discarded.

When comparisons were made between wounded and non-wounded rats, the latter animals were treated exactly like the injured rats with respect to anesthesia, clipping of hair, etc. The procedure differed only in that wounds were not inflicted in the control animals.

Collection of Tissue Samples

At intervals after injury, the wounded rats were reanesthetized

with nembutal. The scab covering the healing wound was removed with a forceps. The healing wound tissue was separated from adhering fascia with a scissors. No adhesions to underlying fascia were observed ten days or longer after wounding. The granulation tissue was then removed.

Samples of skin tissue were obtained by clipping the hair two inches below the lower border of the wound down to the skin. The area was shaven with a razor to remove any residual hair. A piece of skin tissue was excised with a scissors, washed under tap water and blotted. After the removal of liver and muscle (gastrocnemius) tissue the animal was sacrificed.

Liver and early wound tissue were blotted lightly to remove excess blood. All tissues were weighed wet on a Roller-Smith balance directly after removal from the animal. Each sample was immediately placed into an 8" pyrex test tube and twenty-five volumes of acid (20% HCl-50% HCOOH, 1:1) were added. This hydrolyzing mixture was employed as cystine is apparently protected from destruction using hydrochloric and formic acid (Miller, et al., 1937).

The tissues were refluxed for twenty-four hours using cold-finger condensers. After cooling, the samples were made up to 25 ml. with distilled water in volumetric flasks. The hydrolysates were then filtered through Whatman #2 paper and stored in tightly stoppered 6" test tubes. The low pH of the solutions inhibited bacterial growth.

Analysis of Tissue Samples

The tissue samples were analysed for nitrogen, cystine, and methionine.

1. Nitrogen

The micro-Kjedahl method was used for total nitrogen analyses. An aliquot of tissue hydrolysate was digested with concentrated sulfuric acid for approximately forty-five minutes in a micro-Kjedahl flask, and then three drops of 30% hydrogen peroxide were added, and the heating continued for an additional fifteen minutes. This procedure converted the nitrogenous material in the tissue to ammonium sulfate. The digest was then cooled and transferred to a micro-Kjedahl apparatus. This material was finally converted to ammonia gas in the presence of 40% sodium hydroxide, and the ammonia steam-distilled into an aliquot of standardized sulfuric acid. The residual acid was back-titrated with standardized sodium hydroxide in the presence of the indicator methyl red, and the amount of nitrogen in the tissue calculated. Tissue samples were always run in duplicate along with blank determinations. It was found that digestion of samples for longer periods did not increase the recoveries.

2. Cystine (Block, et al., 1948)

Three aliquots of hydrolysate were taken to dryness in vacuo in a hot water bath in order to remove hydrochloric and formic acids. Five ml. of distilled water was added and the clear resulting solution was shaken for one minute. After the addition of five ml. of a saturated solution of sodium bicarbonate, the mixture was again agitated. Two ml. of

phospho-18-tungstic acid (P_2O_5 , WO_4^{2-} ; 1:18) were added to each sample. This was immediately followed by one ml. of distilled water for the blank, and one ml. of a freshly prepared 10% sodium sulfite solution in the case of the duplicate unknowns. The resulting solutions were shaken vigorously for one minute and allowed to stand for eight minutes. The blue colored unknowns along with the blank were read in a Klett-Summerson Spectrophotometer (green filter). The concentration of cystine was obtained from a previously prepared linear calibration curve. The blank determination was considered by subtracting its value from that of the unknowns.

Color development is thought to be due to the reduction of phospho-18-tungstic acid by cysteine. Sodium sulfite is used to reduce cystine to cysteine. It should be pointed out that the blanks remained colorless. This indicated that cysteine was not present in determinable amounts in the tissue studies.

The blue color was found to be stable for one-half hour at room temperature. The range of the procedure was 0.1-1.2 mg. cystine per sample. Recovery experiments using a casein hydrolysate and aliquots of a standard cystine solution gave a maximum error of $\pm 2\%$.

Cystine recovery data are shown in Table III.

3. Methionine (Horn, 1946)

The method used for the determination of methionine in tissue is a modification of the McCarthy-Sullivan (1941) method. The procedure depends upon the production of a cherry-red compound in the presence of methionine and acid nitroprusside. As yet, a rationale for the method

Table III

The Recovery of Methionine and Cystine

Added to Casein Hydrolysates

<u>Hydrolysate</u>	<u>Methionine Found mg.</u>	<u>Methionine Recovered mg.</u>	<u>% Error</u>
Casein	1.16	-	-
Casein + 0.50 mg. methionine	1.64	0.48	1.2
Casein + 1.00 mg. methionine	2.14	0.98	0.9
Casein + 1.50 mg. methionine	2.68	1.52	1.4

<u>Hydrolysate</u>	<u>Cystine Found mg.</u>	<u>Cystine Recovered mg.</u>	<u>% Error</u>
Casein	0.25	-	-
Casein + 0.16 mg. cystine	0.42	0.17	2.0
Casein + 0.32 mg. cystine	0.58	0.33	1.7
Casein + 0.48 mg. cystine	0.73	0.48	0.0

has not been advanced. Accuracy as measured by recovery experiments with a casein hydrolysate and a standard methionine solution was $\pm 3\%$ and the range 0.2-1.2 mg. methionine per sample.

An aliquot of the tissue hydrolysate was mixed with four ml. of 5N sodium hydroxide and one ml. of distilled water. After mixing, 0.1 ml. of a freshly prepared 10% sodium nitroprusside (nitroferrocyanide) was added and the resulting yellow solution shaken for exactly ten minutes. Two ml. of a 3% glycine solution were then added and the clear solution agitated for an additional ten minutes. The concentration of glycine used apparently compensates for variations in color intensity produced at lower amino acid levels. Two ml. of 85% phosphoric acid were finally added slowly with shaking, and the solution was allowed to stand for five minutes. The resulting cherry-red color was read in a Klett-Summerson spectrophotometer (green filter).

Duplicate samples were run on each tissue and the concentration obtained from a linear calibration curve. Blank readings were made by eliminating the nitroprusside from the sample. The value of the blank was subtracted from that of the unknown in calculating methionine concentrations.

Methionine recovery data are shown in Table III.

Determination of S^{35} Labeled Methionine and Cystine in Tissues:

It was thought that the function of methionine and cystine during the healing of experimental wounds might be elucidated by the use of S^{35}

labeled methionine and cystine. Also, metabolic difference between normal and wounded rats might be established with these radioactive substances.

The labeled amine acids were obtained from the Abbott Laboratories. The radioactive compounds were dissolved in a small amount of hydrochloric acid and diluted to volume with distilled water. Aliquots of the material were administered to the animals via intraperitoneal injection at a specified time after wounding. Tissue hydrolysates were analysed for total S^{35} and S^{35} labeled cystine after the administration of DL-methionine S^{35} . Methionine S^{35} was then calculated by difference. In cases where DL-cystine S^{35} was administered to the rats, only total S^{35} determinations were made.

Total S^{35} Determinations in Tissues

Duplicate aliquots of tissue hydrolysate were heated on a hot plate with an equal volume of a nitric-perchloric acid mixture (3 parts concentrated HNO_3 : 1 part 70% $HClO_4$) for twelve hours. 30% hydrogen peroxide was added to the resulting wet ash. This technique served to oxidize sulfur to sulfate (Marsters, 1939). The ash was then taken up in twenty ml. of distilled water and five ml. of a standard sulfuric acid solution was added. The concentration of the acid was such that 60 mg. of carrier barium sulfate was produced upon the addition of barium chloride. The solution was heated for twenty minutes in a water bath (boiling). Ten ml. of a 5% barium chloride solution was added dropwise and agitation of the sample was avoided. The solution was placed back into the water bath, and heating was continued for one-half hour. The cloudy solution was then allowed to cool at room temperature for six hours. Heating and slow cooling

of the solution is thought to insure the formation of large barium sulfate crystals.

The barium sulfate precipitate was finally filtered under suction onto 3 cm. circles of Whatman #42 paper. A stainless steel Tracerlab filtering funnel which is designed for the collection of radioactive precipitates was employed. The precipitate and sides of the funnel were washed with 50% acetone twice, and finally with pure acetone. This treatment prevented creeping of the precipitate. Suction was continued until the precipitate appeared to be dry. After dryness was attained, four drops of a label varnish-acetone mixture (1:10) were added to cement the precipitate to the filter paper. The filter paper was finally mounted on Tracerlab planchets of 2.4 cm. diameter, which prevented curling of the paper, i.e., the edges of the paper were held in place with brass rings.

The described technique insures standard distribution of the barium sulfate over an area of 2.4 cm. diameter. It should be noted particularly that the geometry and backing of each sample was similar.

Partition of S^{35} Labeled Methionine and Cystine in Tissue

Partition of S^{35} labeled methionine and cystine was accomplished by precipitating cystine in the hydrolysates as the copper mercaptide and then determining its activity.

An aliquot of the tissue hydrolysate was taken to dryness in vacuo and the residual material taken up in seven ml. of distilled water. After the addition of two ml. of 1N hydrochloric acid, ten mg. of a standard cystine solution was added (10 mg./ml.). The solution was transferred

to a 50 ml. centrifuge tube and heated to boiling in a water bath. Three ml. of a cuprous oxide suspension (25 mg./ml.) was added with stirring. One minute after the formation of the greenish-gray mercaptide, 0.5 ml. of a 52% sodium acetate solution was added. Sodium acetate served to bring the sample to a pH of 4.0, the point of minimum solubility of the mercaptide. Indicator paper was used to check the hydrogen ion concentration.

The mixture was allowed to stand for forty minutes at room temperature. The sample was finally centrifuged for five minutes and the supernatant decanted. The mercaptide was washed once by suspending in 25 ml. of 95% ethanol for five minutes, then centrifuged again and decanted. Finally, the mercaptide was taken up in hydrochloric acid and treated as in the method for total S^{35} .

The procedure for the precipitation of cystine as the mercaptide was suggested by Zittle and O'Dell (1941).

Determination of S^{35} Activity in Tissue Samples

A Tracerlab Autoscaler was used to determine the activity of the radioactive samples. The sample was placed in a shielded manual sample changer (Tracerlab, SC-90) equipped with a 1.4 mg./cm.² mica window Geiger-Müller tube. Using a standard uranium sample, the operating voltage of the tube was found to be 1.45 kv. Sample geometry was reported to be better than 0.1% when the sample changer was used in determining S^{35} activity.

Diluted aliquots of the S^{35} labeled DL-methionine and DL-cystine

used for injection of the animals were treated in the manner already described for tissue S^{35} , except that 62 mg. rather than 60 mg. of carrier was added. The activity of these samples was used to calculate the percent decay of the radioactive material. All calculations were then extrapolated to zero time (time of S^{35} administration) and 62 mg. barium sulfate. The latter value was used as the tissue samples contain 1.5-3.5 mg. barium sulfate plus 60 mg. carrier barium sulfate. A prepared self-absorption curve indicated that a 2% error was introduced in counting over the range 61.5-63.5 mg. barium sulfate.

Background readings were made before and after each series of samples were analyzed for S^{35} activity. The counts (21 ± 2 /minute) were subtracted from the activity of the samples. Each sample was counted three times; 512 or 1024 counts being made each time. Reproducibility between duplicate samples was approximately 5%.

Analysis of Urine Samples:

In order to understand the influence that injury has on protein metabolism, it was necessary to conduct balance studies. It has already been shown how nitrogen and sulfur balance is calculated. In these studies, urine samples were collected before and after injury in order to determine nitrogen and sulfur excretion. Fecal nitrogen and sulfur analyses were not made as they could be estimated from the excretion of these elements in the urine. Under normal metabolic conditions, it is believed that about 90% of the nitrogen and sulfur excreted by the body is contained in the urine

(Beach, et al., 1942). At regularly scheduled intervals pre- and post-wounding urine samples were collected.

The metabolism cages housing the rats were first washed a number of times with hot tap water and a detergent. The cages were finally washed down with tap water and then distilled water. This procedure rids the cages of essentially all nitrogen and sulfur containing compounds.

The animals were placed back into the metabolism cages and were not disturbed for twenty-four hours. After this length of time the rats were removed from the cages and the cages washed down a number of times with distilled water. The washings, along with the urine already collected in a beaker, were made up to volume in a 100 ml. volumetric flask. The urine was then placed under toluene in a marked, tightly stoppered urine bottle. The urine samples were finally placed in a refrigerator at a temperature of 5° C. until the analyses could be made. Toluene and low temperatures are believed to inhibit bacterial growth in urine. The urine samples were analyzed for total nitrogen, total sulfur, sulfate, and s^{35} labeled sulfur and sulfate.

1. Total Nitrogen

Total nitrogen in urine was analyzed by the micro-Kjedahl method. The procedure has been described previously under tissue analyses.

2. Total Sulfur (Treen and Crutchfield, 1942)

An aliquot of urine was treated with a 3:1 mixture of concentrated nitric and 70% perchloric acids according to the method of Marsters (1939). Details of the digestion procedure were described (total s^{35} in

tissues) in the preceding section.

The digest was taken up in distilled water and two drops of 15% hydrochloric acid were added. The resulting solution was diluted to volume with distilled water and shaken a number of times to insure uniform mixing. Barium sulfate crystals were formed upon the addition of solid barium chloride. The function of the hydrochloric acid was to keep urinary phosphates in solution, and thus prevent their precipitation as barium phosphate. The barium sulfate was estimated turbidimetrically in the Colman Spectrophotometer, and the amount of urinary sulfur determined from a standard linear calibration curve.

In cases where the optical densities of the solutions fell beyond or below the linear segment of the calibration curve, the samples were discarded. Fresh samples of the same urine were then used and appropriate dilutions were made.

All determinations were carried out in duplicate. A blank was run on each urine sample. The blank was treated similarly to the unknown samples, but barium chloride was not added.

3. Total Urinary Sulfate

Total sulfate in the urine was determined by adding hydrochloric acid to three aliquots of urine (1:4) and heating in a water bath for thirty minutes. The samples were then filtered through Whatman #2 paper after diluting to volume. The concentration of sulfate was then measured in the same manner as described for total sulfur.

Determination of S^{35} Labeled Sulfur and Sulfate in Urine

Radioactive samples of urine were treated in the manner indicated in the determination of total urinary sulfur and sulfate. Enough standard sulfuric acid was added to produce a total of 62 mg. of barium sulfate after the addition of 5% barium chloride. The samples were then treated as in the method described for tissue S^{35} . The activity of each sample was finally determined using the Tracerlab Autoscaler.

Duplicate urine samples were used in these determinations.

CHAPTER VII

RESULTS AND DISCUSSION

It has been shown that cystine and methionine, when supplemented to the diet in equivalent amounts, increased the healing rate of wounds in injured animals to the same extent (Williamson, et al., 1952, 1952a). It was postulated that the sulfur containing amino acids might be incorporated into wound tissue protein in relatively large amounts (Fromm, 1952). No data, however, was available to substantiate or refute this hypothesis.

It was thought that because cystine appeared to be the limiting amino acid in a diet devoid of protein, a metabolic product of this amino acid, rather than cystine per se, might be responsible for the accelerated healing of wounds observed in injured animals fed this non essential sulfur amino acid.

Ethionine has been reported to inhibit the uptake of methionine into protein (Simpson, et al., 1950) and also hinder the conversion of methionine to cystine (Goldberg, et al., 1950). An experiment was carried out using ethionine to determine whether the sulfur amino acids function through a protein synthesizing mechanism during the healing of experimental wounds.

Three groups of twenty-four female albino rats each were maintained

on a 5% casein diet for five days prior to wounding. Standard experimental slit wounds were made on the back of the neck as described previously. On the day of wounding, the animals were fed 5% casein diets supplemented with 555 mg. alanine/100 gm. diet (Group I-1), 100 mg. methionine sulfur plus 100 mg. ethionine sulfur/100 gm. diet (Group II-1), and 100 mg. cystine sulfur plus 100 mg. ethionine sulfur/100 gm. diet (Group III-1). The animals were maintained on these diets for the remainder of the experiment, twenty-two days. Alanine was used to compensate for methionine, cystine, and ethionine nitrogen supplied to the rats in Groups II-1 and III-1.

At 8, 15, and 22 day intervals after wounding, one-third of the animals in each group were sacrificed, and the wound strength of a one-half cm. section of regenerating tissue from each rat determined.

The results of the wound strength determinations are displayed in Table IV. Fig. 3 is a plot of this wound strength data against time along with the healing indices. It can be seen from these data that a lower healing rate of wounds is observed in the animals fed Diets II-1 and III-1 than in the control animals (Group I-1). Further, it should be noted that the healing indices are essentially the same for the wounds in the experimental rats (Groups II-1 and III-1). These data may be taken to indicate that ethionine, contained in Diets II-1 and III-1, inhibits the healing rate of wounds in experimentally injured animals. Apparently, the effects of ethionine on wound healing cannot be reversed by equivalent amounts of dietary methionine or cystine.

The decreased rate of wound healing observed in the animals fed

Table IV

The Influence of DL-Ethionine on the
Healing Rate of Wounds

Wound Strength (grams)[†]

Group #	8 Days After Wounding	15 Days After Wounding	22 Days After Wounding	Healing Index
I-1*	245 ± 10	500 ± 14	735 ± 18	35
II-1**	165 ± 8	382 ± 11	512 ± 20	25
III-1***	170 ± 11	380 ± 13	505 ± 15	24

* Diet supplemented with alanine

** Diet supplemented with methionine and ethionine

*** Diet supplemented with cystine and ethionine

† Significance between mean values of wound strength for Groups I-1 and II-1 is "p" = 0.01; between I-1 and III-1, "p" = 0.01

Diet III-1 as compared to the controls, suggests that methionine is required for the healing of wounds. On the other hand, the lowered healing index observed in the Group II-1 rats as compared to the alanine controls (Group I-1) does not necessarily demonstrate the requirement for cystine by the injured rat. It has been shown, however, that cystine accelerates wound healing to the same extent as methionine when added to the diet of wounded rats. Because it has never been established that methionine inhibits the conversion of cystine to its metabolic products, it might be possible to assume that the sulfur amino acids function through a mechanism involving protein synthesis. However, the site of accretion of these amino acids remained to be ascertained.

Four tissues were studied in order to establish the site of amino acid sulfur deposition in the rat after injury. These were wound, skin, liver, and muscle tissue.

In a number of preliminary investigations, wound and skin tissue were analyzed for cystine, methionine, and nitrogen. The data from one of these experiments is presented in Table V. Similar results were obtained in three such studies.

Thirty female albino rats were maintained on a basal 0% protein ration for five days preceding wounding (Expt. 2). After wounding, one group of fifteen rats (Group I-2) was continued on a protein-free diet supplemented with 60 mg. of DL-alanine/100 gm. diet, while the second group of fifteen rats, Group II-2, received a 0% protein diet supplemented with 100 mg. DL-methionine/100 gm. diet. It should be noted that the amino acid

nitrogen contents of both diets were identical.

Circular wounds (4 cm. diameter) were made on the back of the neck of each rat. At intervals of 5, 8, and 11 days after wounding, five animals from each group were anesthetized with sodium nembutal and samples of skin and wound tissue were removed. These tissues were hydrolyzed and then analyzed for methionine, cystine, and nitrogen.

The data displayed in Table V indicates that there is an accretion of the sulfur amino acids as well as nitrogen in the healing wound with time. Paul and his co-workers (1948) demonstrated that the nitrogen content of healing wound tissue approaches that of corresponding unwounded tissue and the two become equal when healing is completed. On the basis of the data presented in Table V, it can be assumed that regeneration has not terminated in the wounds of the rats in Groups I-2 and II-2 eleven days after wounding. However, it should be noted particularly, that the methionine and cystine content of the healing wound tissue exceeds that of normal skin tissue during the experimental period.

Regarding wound tissue, the data shown in Table V indicates that during the earlier periods of regeneration, i.e., five days after injury (about the end of the lag phase), the methionine content of healing tissue has reached a relatively high level as compared to skin. At the end of the experimental period (11 days) the methionine content of wound tissue exceeds that of corresponding unwounded skin tissue. In the case of cystine, this difference is more apparent, i.e., the cystine content of the regenerating wound tissue is approximately 195% (Group I-2) to 235% (Group II-2) greater

Table V

The Cystine, Methionine, and Nitrogen Content
of Wound and Skin Tissue

(% Wet Weight)

Days After Wounding	Wound Tissue			Skin Tissue		
	<u>Cyst.</u>	<u>Meth.</u>	<u>Nit.</u>	<u>Cyst.</u>	<u>Meth.</u>	<u>Nit.</u>
5*	0.25	0.33	1.68	0.31	0.40	3.95
8	0.38	0.42	2.30	0.30	0.42	3.90
11	0.55	0.48	2.95	0.28	0.39	4.02
<hr/>						
	<u>Cyst.</u>	<u>Meth.</u>	<u>Nit.</u>	<u>Cyst.</u>	<u>Meth.</u>	<u>Nit.</u>
5**	0.30	0.37	1.85	0.29	0.44	3.99
8	0.49	0.44	2.36	0.32	0.41	4.00
11	0.70	0.52	3.03	0.30	0.42	3.90

* Group I-2; 0% protein diet supplemented with DL-alanine

** Group II-2; 0% protein diet supplemented with DL-methionine

than that of skin tissue.

By extrapolating the values of methionine and cystine in wound tissue back to the time of wounding, it might be possible to conclude that the accretion of cystine in regenerating tissue progresses slowly during the lag phase and rapidly during the proliferation period. The data in Table V indicates that the converse is true with regard to methionine deposition. It will be shown that data from subsequent studies tend to substantiate this conclusion. It might then be possible to state that there is a synthesis of methionine rich proteins primarily during the lag phase of healing, and of cystine rich proteins principally during the later stages of wound regeneration. Further, it should be noted from the data in Table V that the ratio of wound methionine to cystine decreases with time. This suggests strongly, that, for the most part, the proteins containing these amino acids are quite different.

Previous work (Williamson, et al., 1952, 1952a) demonstrated that injured rats fed a protein-free diet plus methionine exhibited higher wound healing indices than wounded rats fed a protein-free diet supplemented with alanine. The data displayed in Table V indicates that a correlation exists between the healing indices of the animals in Groups I-2 and II-2 and the cystine content of healing wound tissue. This relationship apparently does not hold rigorously in the case of the methionine and nitrogen content of regenerating tissue.

In Fig. 4 is shown a graph of the nitrogen, methionine, and

Fig. 4

cystine content of healing wound tissue plotted against time for this experiment. The healing indices obtained from a comparable study (Williamson, et al., 1952) were 34 for the rats in Group II-2 and 24 in the case of the Group I-2 animals. The slopes of the cystine vs. time curves in Fig. 4 are 5.0 (Group I-2) and 6.8 (Group II-2). The ratio of the healing indices between the two groups is almost identical with the ratio obtained for the slopes of the cystine versus time curves in this experiment. Data from subsequent experiments apparently confirm this empirical relationship. It might be possible then to measure the healing rate of wounds in rats by merely analyzing wound tissue for cystine.

Williamson and his associates (1952) suggested that cystine might be the limiting sulfur amino acid during the healing of experimental wounds in rats. On the basis of the data presented in Table V, this conclusion appears to be valid. This non essential amino acid appears to accelerate the healing process due to the fact that it is required in relatively high concentration in granulation tissue. On the other hand, while methionine is also required by the healing wound tissue per se, in the absence of an adequate dietary source of cystine, the chief function of the former amino acid might be to supply the healing wound with cystine. Further, it should be noted particularly that from the standpoint of chemical composition wound and corresponding unwounded tissue appear to be distinctly different entities.

In the previous experiment it was assumed that the sulfur amino acids, methionine and cystine, as well as wound nitrogen is contained in

protein. It is possible that these substances, especially methionine and cystine, do not exist primarily in proteins but rather in small peptides, e.g., oxidized glutathione in the case of cystine. Twelve female albino rats were used to test this hypothesis. The animals were fed a 10% casein diet during the acclimation and experimental periods. Circular wounds, 1 cm. in diameter, were made in the skin in the region of the back of the neck of each rat. The wounds were allowed to heal for ten days, after which time the granulation tissue was removed and subjected to TCA fractionation and dialysis.

In Table VI is presented data obtained from the fractionation of wound tissue using TCA. In this investigation, it was assumed that twenty-four hour dialysis of the TCA insoluble fraction of wound protein removes any amino acids adsorbed on the surface of the precipitated proteins. The data displayed in Table VI strongly suggests that the sulfur amino acids as well as most of the nitrogen found in wound tissue is contained in proteins. Undoubtedly, small amounts of cystine and methionine exist in non-protein form. However, the techniques employed for the determination of methionine and cystine lacked the sensitivity necessary for such micro-analyses.

Results obtained from the analysis of wound tissue demonstrated the rapid deposition of methionine during the lag phase, and the accretion of cystine rich proteins in wound tissue during the proliferation phase of regeneration. Obviously, the deposition of the sulfur amino acids in wound tissue does not continue indefinitely. However, what occurs with respect

Table VI

TGA Fractionation of Ten Day Old

Wound Tissue*

(% Composition Wet Weight)

Constituents	TGA Insoluble Fraction Dialyzed 24 Hours	TGA Soluble Fraction
Nitrogen	3.11 $\pm$ 0.15	0.15 $\pm$ 0.10
Cystine	0.67 $\pm$ 0.02	0.00
Methionine	0.53 $\pm$ 0.03	0.00

* Animals fed a 10% casein ration

to these amino acids after healing is completed? In order to answer this question, the following experiment was conducted.

Forty female albino rats were maintained on a 6% casein diet for five days prior to wounding. At intervals of 5, 8, 14, 17, 20, 23, 25, 27, 29, and 31 days after injury, four animals were anesthetized with sodium nembutal and samples of wound and skin tissue removed. The animals were then sacrificed. These tissues were weighed, wet, subjected to acid hydrolysis, and finally analyzed for nitrogen, methionine, and cystine. The results of these determinations are shown in Table VII. A graph of percent nitrogen, methionine, and cystine plotted against time for wound tissue in this experiment is presented in Fig. 5.

It should be noted that the results of this experiment are in agreement with those presented previously (Tables V and VI) concerning the methionine, cystine, and nitrogen composition of wound and skin tissue. It can be seen from Fig. 5 that the methionine and cystine concentration of regenerating wound tissue reaches a maximum at approximately twenty-three days after wounding, and then levels off. At thirty-one days after injury, when healing can be considered to be completed, there is no indication that the concentration of the sulfur amino acids in wound tissue will return to the level observed in corresponding unwounded tissue. However, the possibility exists that this may take place later. The nitrogen content of skin and wound tissue, it can be seen from Table VII and Fig. 5, appears to be similar about and beyond the twenty-third day after wounding. These results

Table VII

The Cystine, Methionine, and Nitrogen Content
of Wound and Skin Tissue*

(% Wet Weight)

Days After Wounding	Wound Tissue			Skin Tissue		
	<u>Cyst.</u>	<u>Meth.</u>	<u>Nit.</u>	<u>Cyst.</u>	<u>Meth.</u>	<u>Nit.</u>
5	0.28	0.38	1.63	0.28	0.47	4.38
8	0.35	0.43	2.42	0.28	0.48	4.31
14	0.59	0.51	3.37	0.28	0.42	4.04
17	0.72	0.59	3.42	0.31	0.46	4.13
20	0.80	0.62	3.68	0.33	0.44	3.88
23	0.86	0.67	3.63	0.33	0.46	3.93
25	0.84	0.61	3.86	0.30	0.46	4.01
27	0.80	0.60	3.65	0.32	0.47	3.93
29	0.84	0.65	3.70	0.31	0.46	3.75
31	0.83	0.63	3.78	0.30	0.46	3.64

* Group I-3; 6% casein diet.

Fig. 5

again suggest a fundamental difference between wound and corresponding normal tissue. Further, the data graphed in Fig. 5 substantiates the conclusion that methionine rich proteins are synthesized rapidly in granulation tissue directly after injury, while the converse appears to be true with regard to cystine.

The relatively high concentration of cystine in wound tissue suggests that the cystine-containing proteins are keratin-like in nature. Research on the fractionation of this tissue might possibly establish the nature of the proteins existing in regenerating tissue.

It has been demonstrated that the amino acids, DL-methionine and DL-cystine, when added to the diet of wounded rats caused the wounds in these animals to heal more rapidly than when these sulfur amino acids were not fed (Williamson, et al., 1952, 1952a). Empirical data obtained in the course of balance studies indicated that a correlation exists between sulfur amino acid retention and the healing rate of wounds (Williamson, et al., 1951, 1951a). No such relationship was found to exist in the case of amino acid nitrogen. A mechanism was presented which might, in part, account for the negative nitrogen balance characteristic of trauma (Fromm, 1952). It was suggested that there might be a raiding of "tissue stores" by the wound for methionine and cystine; those amino acids from the degraded proteins not required by the healing wound might be catabolized and the nitrogen excreted in the urine.

Although balance data has been obtained regarding metabolic differences between wounded animals fed different diets, no studies have been

conducted using normal or unwounded rats. In order to determine the physiological state of experimentally injured animals, a study was conducted using wounded and normal animals.

The animals, sixty female albino rats, were fed a 0% protein diet for six days prior to wounding. On the day of wounding and throughout the remainder of the experiment, the animals were fed a 0% protein ration supplemented with 1 mg. cystine per gram of diet. Circular wounds of 4 cm. diameter were made in thirty of the experimental animals (Group II-4). Two days after wounding a tracer dose of S^{35} labeled DL-cystine (1.01×10^6 counts/minute) was administered intraperitoneally to each rat. Twenty-four hour urine samples were collected before wounding and throughout the entire experimental period.

On the 7th, 11th, and 14th days after injury ten rats from each group of thirty animals were reanesthetized with sodium nembutal and samples of wound, skin, liver, and muscle tissue were removed. The animals were then sacrificed. After the usual weighing of the tissue and acid hydrolysis, the samples were analyzed for nitrogen, methionine, cystine, and S^{35} labeled cystine. Total sulfur and sulfate as well as S^{35} labeled sulfur and sulfate determinations were conducted on samples of urine.

Data on the nitrogen, cystine, and methionine content of the tissues analyzed in this study are displayed in Table VIII. It can be seen that the methionine content of wound tissue in the animals in Group II-4 increases significantly from the 7th to the 14th day after wounding. There is apparently no alteration of skin methionine in either normal or

Table VIII

Methionine, Cystine, and Nitrogen Content
of Tissues in Wounded and Normal

Animals

(% Wet Weight)

Days After Wounding	Wound			Skin		
	Meth.	Cyst.	Nit.	Meth.	Cyst.	Nit.
7*				0.472.04	0.302.03	4.122.11
11				0.461.04	0.272.02	4.132.14
14				0.442.04	0.282.03	4.092.26
7**	0.382.05	0.282.03	1.902.09	0.432.02	0.292.01	4.372.19
11	0.411.03	0.512.04	2.312.13	0.392.02	0.272.04	4.132.12
14	0.552.03	0.732.02	2.822.14	0.432.04	0.262.02	4.192.11

* Group I-4; 0% Protein diet supplemented with DL-cystine

** Group II-4; 0% Protein diet supplemented with DL-cystine

Table VIII

Methionine, Cystine, and Nitrogen Content
of Tissues in Wounded and Normal
Animals

(% Wet Weight)

Days After Wounding	Muscle			Liver		
	Meth.	Cyst.	Nit.	Meth.	Cyst.	Nit.
7*	0.69±.05	0.37±.02	3.61±.13	0.65±.04	0.45±.02	3.36±.08
11	0.66±.05	0.39±.05	3.57±.04	0.65±.06	0.47±.02	3.36±.11
14	0.65±.05	0.38±.04	3.76±.12	0.60±.05	0.47±.04	3.27±.16
7**	0.73±.07	0.39±.03	3.74±.07	0.65±.06	0.48±.03	3.37±.10
11	0.66±.03	0.37±.03	3.64±.10	0.51±.04	0.44±.00	3.28±.10
14	0.63±.05	0.37±.01	3.53±.15	0.46±.02	0.45±.03	2.88±.03

* Group I-4; Unwounded

** Group II-4; Wounded

injured rats during this period. The results shown in Table VIII indicate that muscle methionine in the rats in Group II-4 decreases to some extent after injury, however, no significant difference exists in muscle methionine between the experimental and unwounded control animals (Group I-4). However, a significant decrease in muscle methionine can be observed in the wounded rats from the 7th to the 14th day after injury. (See Figure 6.)

It can be seen from the results presented in Table VIII that the level of liver methionine during the experimental period exhibits some decrease in the unwounded rats (Group I-4). This is probably due to the diet fed these animals, which was devoid of protein. On the other hand, there is a marked drop in liver methionine in the wounded animals (Group II-4) from the 7th to the 14th day after wounding. This decreased liver methionine concentration which is significantly different from that observed in the unwounded rats, strongly suggests that the liver makes an appreciable contribution of methionine to the wound.

Further, the data displayed in Table VIII indicates that there is little change in the cystine content of liver, skin, and muscle in either group of animals during the course of the experiment. It might then be concluded that the diet fed the animals, which contains appreciable quantities of DL-cystine, meets at least part of the wound's requirement for this amino acid. (See Figure 7.)

As in previously described experiments, there appears to be a marked and significant increase in wound cystine after injury. The data presented in Table VIII again suggests the formation of methionine rich

Fig. 6

Fig. 7

proteins in early regeneration of wound tissue, whereas cystine abundant proteins appear to be synthesized primarily during the more advanced stages of the healing process, i.e., after the lag phase. Further, it should be noted particularly that the data obtained in this experiment regarding the nitrogen, cystine, and methionine content of wound tissue is similar to that presented in previously described experiments in this thesis. In an earlier study, rats were fed a protein free diet supplemented with DL-methionine (Group II-2), while in this investigation the animals received an equivalent amount of DL-cystine in their 0% protein diet. It can be seen that the cystine content of wound tissue in both groups of animals is similar. These results tend to substantiate the conclusion that the cystine content of granulation tissue may be of use as a criterion of the healing rate of wounds.

Nitrogen analyses (Table VIII) appear to indicate little alteration in this element in muscle and skin with time in the control animals (Group I-4). However, liver nitrogen can be seen to decrease in these same animals from the 7th to the 14th day after wounding. This effect is undoubtedly caused by the protein-free diet fed these rats. The wounds, as shown in Table VIII, increase in nitrogen with time. These results are in agreement with previous data. The nitrogen content of skin and muscle tissue in the Group II-4 rats appears to decrease slightly as compared to the control animals. However, there is a marked and significant decrease in liver nitrogen in the experimentally injured animals. This might be taken to mean that the liver is the primary source of wound methionine

and nitrogen, and is also the source of large quantities of urinary nitrogen.

In Table IX is presented data from two groups of experimentally wounded rats subjected to experimental conditions similar to those for the animals in Groups I-4 and II-4. The animals in Group I-5 were fed a 0% protein diet supplemented with DL-alanine (60 mg./100 gm. diet), while those in Group II-5 received a protein-free diet supplemented with methionine (100 mg./100 gm. diet). Each group of rats was made up of eighteen animals.

The data on the nitrogen, methionine, and cystine content of liver and muscle tissue is displayed in Table IX. It can be seen from these results that the concentration of liver and muscle cystine remains relatively constant after wounding in injured rats (Groups I-5 and II-5). On the other hand, there is a marked decrease in muscle and liver methionine in these same animals. This appears to be true even in the presence of dietary methionine (Group II-5). The results in Tables VIII and IX might then be taken to mean that muscle and liver methionine is a source of wound tissue methionine and/or cystine. On the other hand, most of the cystine contained in these same tissues is not degraded to supply the granulation tissue with cystine. The similar healing indices observed when either methionine or cystine is fed to wounded animals suggests that the wound can utilize the free non essential sulfur amino acid for wound protein synthesis.

In Table X is shown the results obtained on the S^{35} and specific

Table IX

Methionine, Cystine, and Nitrogen Content
of Muscle and Liver Tissue in Wounded Animals

(% Wet Weight)

Days After Wounding	Liver			Muscle		
	Meth.	Cyst.	Nit.	Meth.	Cyst.	Nit.
5*	0.79	0.42	3.40	0.75	0.41	3.44
8	0.59	0.50	3.15	0.74	0.43	3.35
12	0.44	0.47	2.87	0.61	0.39	3.56
5**	0.83	0.45	3.05	0.83	0.41	3.42
8	0.66	0.46	2.71	0.65	0.41	3.61
12	0.47	0.51	2.67	0.63	0.40	3.56

* Group I-5; 0% protein diet supplemented with DL-alanine

** Group II-5; 0% protein diet supplemented with DL-methionine

Table I

Cystine S^{35} ¹ and Specific Activity² of Tissues
In Wounded and Unwounded Animals³

Days After Wounding	Wound		Skin		Muscle		Liver	
	Act.	Sp. Act.	Act.	Sp. Act.	Act.	Sp. Act.	Act.	Sp. Act.
7*			2970± 25	3.72	1970± 90	1.99	7905± 115	6.60
11			2585± 100	3.60	1715± 65	1.65	5965± 120	4.79
14			1740± 110	2.34	1545± 50	1.51	4315± 140	3.48
7**	5475± 125	7.35	3725± 135	4.81	1540± 75	1.48	7650± 135	5.98
11	4560± 80	3.36	2060± 95	2.86	1510± 100	1.52	4695± 130	4.00
14	4350± 80	2.23	1775± 85	2.56	1470± 85	1.49	3445± 75	2.87

1 Calculated in counts/minute/gram of tissue; corrected for decay

2 Specific Activity = $\frac{\text{counts/minute/gram of tissue}}{\text{ng. cystine S/gram tissue}} \times 10^{-3}$

3 Dose of S^{35} labeled cystine is 1.01×10^6 counts/minute/rat

* Group I-4

** Group II-4

activity of the various tissues under discussion in this experiment. From the data presented in Table X, it can be seen that there is a rapid incorporation of S^{35} labeled cystine into the wounds of the animals in Group II-4. Between the 7th and 14th days after wounding there appears to be a 70% decrease in the specific activity of this same tissue. This effect is probably due, primarily, to the accretion of cystine and to a lesser extent, protein turnover.

The S^{35} data displayed in Table X regarding skin tissue indicates that a more rapid loss of this material occurs in the skin of wounded as compared to noninjured rats (Group I-4). Muscle studies (Table X) suggest that a greater amount of S^{35} labeled cystine is incorporated into the muscle protein of normal rats than from those found in Group II-4. Further, loss of the label appears to be more pronounced in the Group I-4 than Group II-4 animals with the passage of time.

The most noticeable alteration in the S^{35} content of tissues occurs in the liver. This can be seen from the data presented in Table X. Further, the greater decrease in activity occurs in the wounded as contrasted with the normal rats in Group I-4. Because the cystine content of liver remains relatively constant, the loss of the label in this tissue must be attributed to protein turnover.

The loss of S^{35} cystine from the tissues as depicted in Table X is probably due primarily to protein turnover and label dilution by dietary DL-cystine in the case of the rats in Group I-4. The difference in activity exhibited between these animals and those in Group II-4 must be

attributed to wounding. These data then suggest that, in general, sulfur metabolism is stimulated in the wounded rats as compared to the control animals.

In Table XI are displayed data concerning the excretion of sulfur, sulfate, neutral sulfur, and nitrogen of the rats in Groups I-4 and II-4. It can be seen from these data that the animals in Group I-4 excrete increasing amounts of sulfate after the addition of dietary cystine (Figure 8). This value reaches its peak on about the 7th day after wounding and then decreases. The excretion of sulfate in the wounded animals (Group II-4) parallels that of the control rats, but at a higher level; 40% greater. These results suggest that wounded animals have the ability to utilize sulfur from cystine to a greater extent than normal rats.

It can be seen from the data in Table XI and Figure 9 that there appears to be a similarity in total sulfur excretion in both groups of rats, i.e., a slight decrease in total sulfur excretion with time. Further, these results indicate that there is a slightly greater excretion of total sulfur in the Group II-4 animals as contrasted with those in Group I-4. Similar conclusions can be drawn from the data regarding sulfur balance in this experiment. The data concerning neutral sulfur in Table XI and Figure 10 indicates a greater excretion of this material in the control animals than in the Group II-4 rats over the course of the experimental period. These data on sulfate and neutral sulfur excretion might be taken to mean that the wounded animals are capable of metabolizing larger amounts of dietary cystine than unwounded animals.

Table XI

Excretion of Nitrogen, Sulfur, Sulfate, and Neutral Sulfur
in Wounded and Unwounded Rats

(Mg./Rat/Day)

Days After Wounding	Nitrogen		Sulfate		Total Sulfur		Neutral Sulfur	
	*	**	*	**	*	**	*	**
1	53.4	57.8	0.73	0.90	2.11	3.09	1.38	2.19
2	42.3	53.5	0.47	0.65	4.59	5.78	4.12	5.13
3	47.4	48.6	0.73	1.27	4.75	5.07	4.02	3.80
4	33.4	46.7	0.92	1.36	4.37	4.35	3.45	2.99
5	32.0	48.6	0.99	1.56	3.95	4.03	2.96	2.47
6	32.5	38.3	1.04	1.68	4.85	4.93	3.81	3.25
7	30.8	38.0	1.07	1.10	4.55	4.70	3.48	3.60
8	38.6	43.5	0.40	0.53	4.13	4.05	3.73	3.52
9	34.9	44.6	0.28	0.32	3.87	3.53	3.59	3.21
10	37.4	44.6	0.36	0.68	4.20	3.70	3.84	3.05
11	31.8	37.4	0.52	0.71	4.10	4.26	3.58	3.45
12	39.2	39.2	0.64	0.72	4.00	3.60	3.36	2.88
13	32.9	37.7	0.62	0.80	4.00	4.10	3.38	3.30

* Group I-4; Unwounded Rats

** Group II-4; Wounded Rats

Fig. 8

Fig. 10

It can be seen from the data concerning nitrogen excretion in Table XI that the peak of the nitrogen loss from the animals in Group I-4 occurs at about the third day after the addition of cystine to the diet. On the other hand, the leveling off of nitrogen excretion does not occur in the wounded animals until after the fifth day following injury. It should be noted particularly from the data in Table XI that the excretion of nitrogen is markedly greater in the wounded rats than in the unwounded animals (Group I-4). The average nitrogen:total sulfur excretion ratio is approximately 7.6 (Group I-4) and 10.5 (Group II-4) (Figure 11). This again suggests that there is a greater utilization of amino acid sulfur in the body of the injured rat as compared to the unwounded rat.

In Table XII is shown data on the excretion of S^{35} labeled total sulfur and sulfate. It can be seen that these results substantiate those presented in Table XI regarding total sulfur and sulfate.

In the experiment described above, it was shown how S^{35} labeled DL-cystine helped elucidate the function of cystine during the healing of experimental wounds. It was thought that a better insight into the function of the sulfur amino acids in experimental wound healing might be obtained by following the metabolism of both S^{35} methionine and cystine simultaneously.

In the experiment to be described, sixty female albino rats were maintained on a protein-free diet for three days prior to wounding. On the day of wounding, the animals were divided into four groups of fifteen

Fig. 11

Table XII

The Excretion of S^{35} Labeled Total Sulfur and Sulfate
in Wounded and Unwounded Rats*

(Counts/Minute/Rat/Day)

Days After Wounding	Total Sulfur		Total Sulfate	
	**	***	**	***
2	32180	35920	4960	10800
3	22700	26500	9160	13020
4	15700	18160	6840	9540
5	14320	17240	6660	9180
6	12860	14700	5940	7560
7	12500	13860	6500	7000
8	7080	7760	3560	3440
9	6600	6960	2880	3260
10	6980	7920	3260	4400
11	6700	6960	3260	3840
12	6940	6640	3900	3440
13	5980	5800	3440	3700

* Dose DL-cystine S^{35} 1.01×10^6 counts/minute/rat; Corrected for decay.

** Group I-4; Unwounded rats

*** Group II-4; Wounded rats

rats each. Two groups of animals were wounded (Groups II-6 and IV-6), while the other two groups served as controls (Groups I-6 and III-6). Four days after wounding, each rat in Groups I-6 and II-6 was injected intraperitoneally with a tracer dose of S^{35} labeled methionine (5.90×10^5 c/m), while each animal in Groups III-6 and IV-6 received a tracer dose of DL-cystine S^{35} (6.66×10^5 c/m). On the 5th, 9th, and 13th days after wounding one-third of the animals from each group were subjected to sodium nembutal anesthesia and samples of wound, skin, muscle, and liver tissue were removed. After sacrificing the rats, the tissues were analysed for cystine, methionine, and S^{35} labeled methionine and cystine. Urine samples were collected from the animals from the 5th through the 11th day after wounding. These samples were analysed for S^{35} labeled total sulfur and sulfate.

Data is presented in Table XIII on the concentration of methionine and cystine in wound, skin, muscle, and liver tissue in this experiment. It can be seen that the results obtained in this study are in agreement with those presented in the previously discussed experiments.

Tables XIV and XV indicate the data on the uptake of methionine and cystine S^{35} into rat tissues after the administration of S^{35} labeled DL-methionine. It can be seen from these data that the incorporation of radioactive methionine into the wounds of injured animals (Group II-6) is very rapid. Further, the loss of this amino acid from regenerating wound tissue from the 5th to the 13th day after injury is quite pronounced. The results displayed in Table XIV also indicate that the uptake and loss

Table XIII

Cystine and Methionine Content of Tissues
in Wounded and Unwounded Animals

(% Wet Weight)

Days After Wounding	Wound		Skin		Muscle		Liver	
	Cyst.	Meth.	Cyst.	Meth.	Cyst.	Meth.	Cyst.	Meth.
5*	0.24±.03	0.32±.03	0.26±.01	0.35±.04	0.33±.02	0.54±.01	0.42±.02	0.79±.04
9	0.38±.03	0.40±.02	0.26±.03	0.34±.04	0.34±.04	0.79±.03	0.43±.03	0.70±.02
13	0.58±.04	0.47±.03	0.25±.03	0.35±.03	0.33±.03	0.75±.02	0.43±.03	0.62±.02
5**			0.28±.02	0.37±.02	0.34±.04	0.62±.03	0.43±.01	0.78±.04
9			0.25±.03	0.37±.01	0.33±.02	0.51±.05	0.40±.00	0.75±.04
13			0.24±.02	0.34±.03	0.32±.02	0.53±.02	0.41±.03	0.71±.04

* Groups II-6 and IV-6; Wounded rats

** Groups I-6 and III-6; Unwounded rats

Table XIV

Methionine S³⁵* and Specific Activity of Tissues

in Wounded and Unwounded Animals

After DL-Methionine S³⁵**

Administration

Days After Wounding	Wound		Skin		Muscle		Liver	
	Act.	Sp. Act.	Act.	Sp. Act.	Act.	Sp. Act.	Act.	Sp. Act.
5 ^a	3630±110	5.30	735±30	0.98	475±40	0.26	5350±200	3.15
9	2420±85	2.82	670±30	0.92	855±65	0.90	4340±175	2.83
13	1375±60	1.35	510±35	0.68	610±80	0.38	3470±110	2.62
5 ^b			425±35	0.53	545±45	0.31	4750±85	2.82
9			415±45	0.52	565±50	0.33	4550±90	2.82
13			560±20	0.77	650±70	0.36	3940±85	2.57

a Group II-6; Wounded Rats

b Group I-6; Unwounded Rats

* Calculated in counts/minute/gram of tissue; corrected for decay

** Dose of S³⁵ labeled methionine (DL-) is 5.90×10^5 c/m; corrected for decay

Table XV

Cystine S³⁵* and Specific Activity of Tissues

in Wounded and Unwounded Animals

After DL-Methionine S³⁵**

Administration

Days After Wounding	Wound		Skin		Muscle		Liver	
	Act.	Sp. Act.	Act.	Sp. Act.	Act.	Sp. Act.	Act.	Sp. Act.
5 ^a	2630±95	4.10	575±45	0.83	520±40	0.59	5000±210	4.46
9	2430±110	2.40	685±60	0.99	485±35	0.53	3140±160	2.76
13	2840±75	1.83	690±75	1.03	570±70	0.65	2980±190	2.61
5 ^b			525±40	0.70	570±35	0.63	5020±175	4.40
9			545±40	0.82	590±80	0.67	3060±150	2.86
13			655±35	1.02	625±60	0.73	3100±110	2.84

a Group II-6; Wounded rats

b Group I-6; Unwounded rats

* Calculated in counts/minute/gram of tissue; corrected for decay

** Dose of DL-Methionine is 5.90×10^5 c/m; corrected for decay

of methionine S^{35} is greater in skin, muscle, and liver tissue in wounded rats (Group II-6) than in uninjured animals (Group I-6). These data appear to suggest rather strongly that methionine metabolism is accelerated in the wounded animal as contrasted with normal animals.

The data presented in Table XVI indicates that the uptake by wounds of cystine S^{35} after the administration of S^{35} labeled methionine is slower than that of radioactive methionine itself. This is to be expected as the formation of cystine is dependent upon the breakdown of methionine; further, during this period there is a great demand for methionine by healing wound tissue. It should be noted from these results on wound cystine S^{35} that the uptake of this amino acid into wound proteins remains relatively constant from the 5th through the 13th day after wounding. These results appear to contradict those presented for the S^{35} labeled cystine content of wounds in rats in Group II-4. However, it should be remembered that cystine derived from methionine is retained for longer periods of time within the body than cystine supplied per se. In other words, "potential" cystine, i.e., in the form of methionine, can be utilized more efficiently for protein synthesis than cystine itself. This conclusion appears to be substantiated by urine S^{35} data from this experiment (Table XVII).

In Table XVI results are presented on the uptake of S^{35} labeled cystine into the tissues of wounded (Group IV-6) and unwounded control animals (Group II-6) after the administration of radioactive DL-cystine. The data provided in this table indicates little difference with respect to liver, skin, muscle tissue regarding cystine S^{35} uptake and loss in

Table XVI

Cystine S^{35} * and Specific Activity of Tissue
in Wounded and Unwounded Animals
After DL-Cystine S^{35} **
Administration

Days After Wounding	Wound		Skin		Muscle		Liver	
	Act.	Sp. Act.	Act.	Sp. Act.	Act.	Sp. Act.	Act.	Sp. Act.
5 ^a	6030	9.40	1580	2.32	1045	1.19	9850	8.80
9	6500	6.40	1720	2.51	1220	1.35	6950	6.10
13	5400	3.48	1570	2.36	1250	1.42	5750	5.05
5 ^b			1875	2.52	1030	1.14	10750	9.45
9			1825	2.74	1225	1.39	7310	6.86
13			1555	2.43	1310	1.54	5600	5.14

a Group IV-6; Wounded rats

b Group III-6; Unwounded rats

* Calculated in counts/minute/gram of tissue; corrected for decay

** Dose of DL-Cystine is 6.66×10^5 c/m; corrected for decay

Table XVII

Urinary Excretion of S^{35} Labeled Total Sulfur, Sulfate,
and Neutral Sulfur in Wounded and Unwounded Rats^a

(Counts/Minute/Rat/Day)

Group #	Excrement	5th	6th	7th	8th	9th	10th	11th
(Days After Wounding)								
I-6	<u>Sulfate</u>	4680	2940	2710	2290	1950	1880	1785
II-6		4840	3190	4100	2500	2750	2670	2230
III-6		5530	5110	4600	3900	2530	2760	2630
IV-6		5300	5600	7450	4500	4550	4550	4430
I-6	<u>Total Sulfur</u>	18700	6180	7300	5230	5100	5000	4520
II-6		19400	7900	6920	5250	5660	4100	4300
III-6		20750	10675	9135	8240	5540	6840	6080
IV-6		22650	11550	11300	8600	7210	8190	6870
I-6	<u>Neutral Sulfur</u>	14020	3240	4590	2940	3150	3120	2735
II-6		14560	4410	2820	2750	2910	1430	2070
III-6		15220	5565	4535	4340	3010	4080	3450
IV-6		17350	6050	3850	4100	2660	3640	2440
Unwounded*		850	2170	1890	1610	580	880	845
Wounded*		460	2110	3350	2000	1800	1800	2200

^a Corrected for dose difference of S^{35} labeled meth. and cyst., and decay

* Rate of methionine $\rightarrow$ cystine/unit time

wounded and uninjured rats. The results concerning liver tissue appear to disagree with those presented in Table X. However, it should be remembered that label dilution with dietary cystine is not a factor in this experiment. The data concerning wound tissue in Table XVI is similar to that presented in Table X, however, these results indicate that the peak of S^{35} labeled cystine uptake into wound tissue proteins occurs between the 2nd and 4th day after the injection of DL-cystine S^{35} .

A better understanding of protein metabolism can be obtained using radiotracers when specific activities are considered. For these studies specific activity was defined as:

$$\text{Specific Activity} = \frac{(\text{Activity-c/min/gm. tissue})}{\text{mg. S/gm. tissue}} \times 10^{-3}$$

When specific activity is plotted against time, such phenomena as replacement and an indication of protein turnover can be obtained. Unfortunately, most of the data presented in this thesis cannot be used in the calculation of protein half-lives. This is due to the fact that the half-lives of skin, muscle, and liver tissue exceed the experimental period in these studies. However, in the case of wound cystine and methionine, such data are available. Fig. 12 that the average half-life of wound proteins containing methionine in rats fed a protein-free diet is approximately 5.3 days, while those containing cystine is about 7.2 days. These data suggest that the turnover of methionine rich proteins in wound tissue during the relatively early stages of regeneration is greater than that of the cystine wound proteins. It is interesting to note that the half-lives of cystine containing wound proteins appears to be independent of the source of this

Fig. 12

amino acid, i.e., whether it is derived from methionine or not.

Results concerning the analyses of rat urines in this experiment are presented in Table XVII. These data were corrected for dose differences. It can be seen from the data shown regarding the excretion of S^{35} labeled sulfate, that wounded animals (Groups II-6 and IV-6) excrete larger amounts of sulfate S^{35} than do unwounded rats. These data suggest an increased catabolism of the sulfur containing amino acids in injured animals as compared to normals (Groups I-6 and III-6).

Because the conversion of methionine to cystine is a prerequisite for the degradation of the former amino acid to sulfate, it may be possible to calculate rates of conversion of methionine to cystine in this experiment. In other words, in the reaction; methionine $\longrightarrow$ cystine $\longrightarrow$ sulfate, if the number of molecules of methionine and cystine converted to sulfate at a definite time interval after the administration of the two amino acids is known, it may be possible to calculate the amount of methionine converted to cystine during this period. If this hypothesis is valid, the data in Table XVII on S^{35} labeled sulfate can be used in this determination. The results concerning these calculations in Table XVII indicate that four days after injury the conversion of methionine to cystine is inhibited in the wounded rat as compared to the unwounded animal. By about the fifth day the conversion rates are quite similar. After this time, however, more cystine is formed from methionine in the injured than control animal. These results regarding sulfate excretion are commensurate with those concerning wound tissue. It appears that during the earlier stages of healing (lag phase) there is a demand for methionine by regenerating tissue, while

in the later stages of this process cystine rich proteins are formed primarily.

Total sulfur S^{35} excretion data are displayed in Table XVII.

These data demonstrate that the injured animals in Groups II-6 and IV-6 excrete slightly more S^{35} labeled sulfur than do unwounded rats. Further, more cystine sulfur is lost from the body than is methionine sulfur. These results may then be taken to mean that there is a greater utilization of methionine than cystine by the rat. This effect is probably due to the fact that methionine is retained for longer periods of time by the rats than is cystine.

Neutral sulfur S^{35} excretion data, obtained by difference from the results of S^{35} labeled total sulfur and sulfate are shown in Table XVII. These data indicate that wounded animals excrete less S^{35} labeled methionine and cystine than do unwounded rats (Groups I-6 and III-6); i.e., the injured animal can utilize the sulfur amino acids more efficiently than unwounded animals.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

Female albino rats were used to study the effect of the sulfur containing amino acids on the healing of experimental skin wounds in rats.

Although it had been shown that methionine and cystine could accelerate the healing of wounds in animals fed a protein-free diet, no mechanism to account for this effect had been suggested. The sulfur amino acids appear to be unique in this respect, as other amino acids, both "essential" and "non-essential" do not appear to influence the healing rate of wounds in animals fed a diet devoid of protein.

It was stressed in the introduction of this thesis that the regenerating wound cell is limited in its growth and proliferation by its intracellular composition. Once the wound's requirement for lipids, carbohydrates, minerals, vitamins, and the sulfur amino acids has been met, other amino acids become limiting. Further, the results obtained in this thesis concern regenerating skin tissue, and, therefore, these results cannot necessarily be projected to other tissues.

Wound, skin, muscle, and liver tissue in wounded and unwounded animals were analyzed for cystine, methionine, and nitrogen. ^{35}S labeled methionine and cystine was employed to elucidate the function of the

sulfur containing amino acids during the healing of experimental wounds. From the results of the experiments described in this thesis, the following conclusions were arrived at:

Ethionine, when fed to wounded rats, inhibits the healing of wounds in these animals. This is true even in the presence of equivalent amounts of methionine and cystine. These results suggest that a metabolic product of cystine was not the cause of accelerated healing observed in wounded rats fed this amino acid. It was concluded, therefore, that the sulfur amino acids, methionine and cystine, function through a mechanism involving protein synthesis.

Analysis of wound and skin tissue in wounded rats, indicated that there is an accretion of methionine, cystine, and nitrogen in regenerating wound tissue with time. It was found that after the lag phase of wound healing, the cystine and methionine content of wound tissue exceeds that of corresponding unwounded tissue.

Extrapolation of the data concerning the methionine and cystine content of wound tissue, suggests the formation of methionine rich proteins occurs primarily during the earlier stages of the healing process, i.e., during the lag period. Further, with regard to cystine, the cystine abundant wound proteins are accumulated in wound tissue principally during the proliferation phase of regeneration.

Data was presented which, empirically, suggests a correlation between the healing rate of wounds and the cystine concentration of this tissue. It might be possible then, to evaluate healing on this basis.

Methionine and cystine contained in wound tissue probably exist in proteins, rather than in small peptides or as free amino acids.

Analysis of wound tissue from the lag to the maturation phase of healing, i.e., when healing is completed, indicates that wound tissue contains about 50% more methionine, and 280% more cystine than corresponding unwounded tissue. The nitrogen content of both tissues were almost identical at the maturation phase of healing. Further, there was no indication that the level of the sulfur amino acids in scar tissue begins to approach that of normal tissue after healing is "completed".

Chemically, skin and corresponding wounded tissue are distinctly different entities.

Analysis of muscle and liver tissue indicates that both these tissues supply the healing wound with methionine. However, of the two tissues, the liver appears to make the greater contribution per gram of tissue. The cystine content of liver and muscle tissue remains the same after experimental injury. This suggests that there is a conservation of the muscle and liver cystine containing proteins after injury.

S^{35} labeled methionine was found to be incorporated more rapidly into healing wound tissue than cystine S^{35} when S^{35} labeled methionine was administered to the injured rat. However, the activity of the radiocystine remained at a higher level for a longer period of time than did methionine activity.

The average half-lives of methionine and cystine containing wound proteins were calculated. It was found that 50% of the methionine

containing wound proteins are replaced in approximately 5.3 days, while cystine containing proteins have a half-life of 7.2 days. Further, there is a stimulation of sulfur metabolism in general in wounded animals as contrasted with uninjured rats.

Analysis of urine from wounded and unwounded rats suggests that the injured animal catabolized more amino acid sulfur than do normal rats.

Although wounded animals excrete slightly more amino acid sulfur than do unwounded rats, there appears to be a greater utilization of methionine and cystine by the wounded animal as contrasted with the uninjured rat. Also, the nitrogen:sulfur excretion ratio is greater in the latter animal than in the wounded rat. This further indicates that injured animals require more methionine and cystine than normal animals.

The data obtained using radioactive sulfur amino acids appears to indicate that there is an increased conversion of methionine to cystine in the experimentally wounded rat.

Finally, the sulfur amino acids probably accelerate the healing rate of wounds in injured animals due to the fact that they are required in relatively high concentration for the synthesis of wound protein.

CHAPTER IX

BIBLIOGRAPHY

- Allison, J. B., Anderson, J. A., and Seeley, R. D. (1946) Determination of Nitrogen Balance Index in Normal and Hypoproteinemc Dog, Ann. New York Acad. Sci., 47, 245-271
- Allison, J. B., Anderson, J. A., and Seeley, R. D. (1947) Some Effects of of Methionine on the Utilization of Nitrogen in the Adult Dog., J. Nutrition, 33, 361-370
- Allison, J. B. (1949) Biological Evaluation of Proteins, Advances in Protein Chemistry, V, 155-200
- Almquist, H. J. and Grau, C. R. (1945) Further Studies on Cystine, Methionine, and Choline in Chick Diets, J. Nutrition, 29, 219-222
- Awapara, J. (1953) -Aminoethanesulfinic Acid: An Intermediate in the Oxidation of Cysteine in Vivo, J. Biol. Chem., 203, 183-188
- Awapara, J. and Wingo, W. J. (1953a) On the Mechanism of Taurine Formation From Cysteine in the Rat, J. Biol. Chem., 203, 189-194
- Baker, B. L. and Whitaker, W. L. (1950) Interference with Wound Healing by the Local Action of Adrenocortical Steroids, Endocrinol., 46, 544-551
- Barnes, T. C. (1945) Further Studies on the Phase-boundary Potential of Acetylcholine, Federation Proc., 3, 65
- Barnes, T. C. (1946) Electric Measurement of Healing Rate of Human Skin, Hahnemann. Monthly, 81, 8-10
- Barnes, T. C. (1951) Am. J. Med. Sciences, 222, 601-603
- Barnes, T. C., Krasic, J., and Amoroso, M. D. (1951a) Further Studies of the Rate of Healing of Human Skin Measured by the Electric Wound Potential of Experimental Abrasions, Am. J. Surg., 82, 720-726

- Barnes, T. C. (1952) An Electrical Method of Assaying the Effects of Drugs on the Wound Potential of Skin, Federation Proc., 11,320
- Barnes, T. C. (1953) Factors in Electrical Method of Measuring the Rate of Wound Healing, Federation Proc., 12,300
- Barnett, R. J. (1953) The Histochemical Distribution of Protein-bound Sulfhydryl Groups, J. Natl. Cancer Inst., 13, 905-925
- Beach, E. F., Teague, D. M., Hoffman, O. D., Munks, B., Hummel, F. C., Williams, H. H., and Macy, I. C. (1942) The Sulfur Metabolism of Children, J. Nutrition, 24, 257-270
- Binkley, F. (1944) Cleavage of Cystathionine by an Enzyme System from Rat Liver, J. Biol. Chem., 155, 39-43
- Binkley, F. and Okeson, D. (1950) Purification of the Enzyme Responsible for the Cleavage of Cystathionine, J. Biol. Chem., 182, 273-277
- Binkley, F. (1951) Biological Transfer of Sulfur, Div. of Biol. Chem., Am. Chem. Soc., Abstracts, p 1c
- Binkley, F. (1952) Pyridoxine and the Transfer of Sulfur, J. Biol. Chem., 194, 109-113
- Blaschko, H. (1942) 1(-)-Cysteic Acid Decarboxylase, Biochem. J., 36, 571-574
- Block, R. J. and Bolling, D. (1948) The Determination of the Amino Acids Burgess Pub. Co., Minneapolis
- Blunt, J. W., Platz, C. M., Lattes, R., Howes, E. L., Meyer, K., and Reagen, C. (1950) Effect of Cortisone on Experimental Fractures in the Rabbit, Proc. Soc. Exper. Biol. and Med., 73, 678-681
- Borsook, H. (1950) Protein Turnover and Incorporation of Labeled Amino Acids Into Tissue Proteins, Physiol. Revs., 30, 206-219
- Borsook, H., and Keighley, Proc. Roy. Soc. London, B118, 448 (1935)
- Borsook, H., Deasy, G. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H. (1950) Incorporation in Vitro of Labeled Amino Acids Into Rat Diaphragm Proteins, J. Biol. Chem., 186, 309-315
- Bosse, M. D. and Axelrod, A. E. (1948) Wound Healing In Rats with Bistin, Pyridoxin, or Riboflavin Deficiencies, Proc. Soc. Expl. Biol. and Med., 67, 418-421

- Bourne, G. (1944) Effect of Vitamin-C Deficiency on Experimental Wounds, Lancet, 1, 688-691
- Bradfield, J. R. G. (1951) Glycogen of Vertebrate Epidermis, Nature, 167, 40-41
- Brush, M. K., Wielman, W., and Swanson, P. P. (1947) Amino Acids in Nitrogen Metabolism With Particular Reference to the Role of Methionine, J. Nutrition, 33, 389-410
- Carmon, P. R., Steffee, C. H., Frasier, L. J., Rowley, D. A., and Stepto, R. J. (1947) The Influence of Time of Ingestion of Essential Amino Acids Upon Utilization in Tissue Synthesis, Fed. Proc., 6, 390
- Carrouthers, G. and Surtzeff, V. (1953) Biochemistry and Physiology of Epidermis, Physiol. Revs., 33, 229-243
- Chalkey, H. W., Algire, G. H., and Morris, H. F. (1945) Effect of the Level of Dietary Protein on Vascular Repair in Wounds, J. Natl. Cancer Inst., 6, 363-372
- Charney, J., Williamson, M. B., Bernhard, F. W. (1947) Apparatus for Determining Tensile Strength of Healing Wounds, Science, 105, 396-399
- Chatagner, F. and Bergert, B. (1951) Decarboxylation Enzymatique, In Vitro Et In Vivo, De L'acide -Cysteinesulfinique Dans Le Foie Des Animaux Superieurs, Compt. Rend. Acad., 232, 448-450
- Clark, A. H. (1919) The Effect of Diet on the Healing of Wounds, Bull. Johns Hopkins Hosp., 30, 117-120
- Cope, O., Nathanson, I. E., Bourke, G. M., and Wilson, H. (1943) Metabolic Observations, Ann. Surg., 117, 937-958
- Corley, R. C., Wolf, P. A., and Nielson, E. K. (1938) The Amino Acids Essential for the Adult Animal, J. Biol. Chem., xxvi
- Co Tai, C., Wright, A. M., Mulholland, I., Barchan, I., Breed, E. S. (1944) Nutritional Care of Cases of Extensive Burns, Ann. Surg., 119, 815-823
- Co Tai, C. (1945) The Value of Protein and Its Chemical Components in Surgical Repair, Bull. N. Y. Acad. Med., 21, 629-655
- Cox, W. M., Mueller, A. J., Elman, R., Albanese, A. A., Kemmerer, K. S., Barton, R. W., and Holt, L. E. (1947) Nitrogen Retention on Rats, Dogs, and Man; The Effect of Adding Methionine to an Enzymic Hydrolysate of Casein, J. Nutrition, 33, 437-457

- Croft, P. B. and Peters, R. A. (1945) Effect of Methionine Upon Nitrogen Losses in Urine Following Severe Burns, Nature London, 155, 175
- Croft, P. B. and Peters, R. A. (1945a) Nitrogen Loss After Thermal Burns; Effect of Adding Protein and Methionine to the Diet of Rats, Lancet, 1, 266-271
- Cucinatta, U. (1948) Acta Vitaminol., 2, 108-110
- Cucinatta, U. (1951) Sull'azione della vitamina B₂, della vitamina B₆ e dell'acido pantotenico sulla guarigione della ferite, Acta Vitaminol., 5, 202-205
- Cuthbertson, D. P. (1929) The Influence of Prolonged Muscle Rest on Metabolism, Biochem. J., 23, 1328-1345
- Cuthbertson, D. P. (1946) Proc. Nat. Soc., 4, 185
- Cuthbertson, D. P., McGirr, J. L., and Robertson, J.S.M. (1939) Effect of Fractures of Bone on Metabolism of Rat, Quart. J. Exper. Physiol., 29, 13-25
- Davidson, J. H. (1945) Biochemistry of Wound Healing, Brit. Med. Bull., 3, 73, 685
- du Vigneaud, V., Chandler, J. P., Mayer, A. W., and Kepple, D. M. (1939) The Effect of Choline on the Ability of Homocystine to Replace Methionine in the Diet, J. Biol. Chem., 131, 57-76
- du Vigneaud, V., Chandler, J. P., Cohn, N., and Brown, C. B. (1940) The Transfer of the Methyl Group from Methionine to Choline and Creatine, J. Biol. Chem., 134, 787-788
- du Vigneaud, V., Kilmer, G. W., Rachelle, J. E., and Cohn, M. (1944) On the Metabolism of the Conversion in Vivo of Methionine to Cystine, J. Biol. Chem., 155, 645-651
- Eckert, J. H. (1948) Sulfur Balance Indexes of Casein in Adult Dogs With and Without Addition of DL-Methionine, Arch. Biochem., 19, 379
- Emery, F. E. (1938) Studies on Hypertrophy, Regeneration, and Retardation of Ovarian Weights in Growing Rats After Esterone Injections, Quart. J. Exp. Physiol., 27, 17-26
- Fisher, A. (1946) Biology of Tissue Cells, Cambridge
- Forkner, L., Chaikoff, I. L., Entman, C., and Tarver, H. (1951) Oxidation of Methionine S³⁵ by the Eviscerated Dog, J. Biol. Chem., 188, 31-35

- Friedberg, F., Farver, H., and Greenberg, D. M. (1948) The Distribution Pattern of Sulfur-Labeled Methionine in the Protein and the Free Amino Acid Fraction of Tissues After Intravenous Administration, J. Biol. Chem., 173, 355-361
- Friedgood, C. E., Vars, H. M., and Zarbe, J. W. (1950) Influence of Fat in the Diet Upon Nitrogen Balance and Liver Regeneration, Amer. J. Physiol., 163, 347-363
- Fromgeodt, C. (1939) Sur la degradation anaerobie de la l-cystine par la desulfurase, nouveau ferment contenu dans le foie, Compt. rend. 209, 1019-1021
- Fromm, H. J. (1952) Nitrogen and Sulfur Metabolism During the Healing of Experimental Wounds, Master's Thesis, Loyola University, Chicago
- Fruton, J. S. and Simmonds, S. (1953) General Biochemistry, John Wiley & Sons, Inc.
- Geiger, E. (1947) Experiments With Delayed Supplementation of Incomplete Amino Acid Mixtures, J. Nutrition, 34, 97-111
- Geiger, E. (1948) The Role of the Time Factor In Feeding Supplementary Proteins, J. Nutrition, 36, 813-819
- Gircoud, A. and Leblond, C. P. (1951) The Keratinization of Epidermis and Its Derivatives, Ann. N. Y. Acad. Sci., 53, 613-626
- Goldberg, R. C., Chaikoff, I. L., and Dodge, A. H. (1950) Destruction of Pancreatic Acinar Tissue by DL-Ethionine, Proc. Soc. Expt. Biol. and Med., 74, 869-872
- Grandon, J. H., Lund, C. C., and Mill, D. B. (1940) Experimental Human Scurvy, New Engl. J. Med., 223, 353-369
- Green, J. W., Hearn, C. E., and Allison, J. E. (1953) Effect of Diet of Wound Healing in Rat Muscle, Fed. Proc., 12, 415
- Harvey, S. C. and Howes, E. L. (1930) Effect of High Protein Diet on the Velocity of Growth of Fibroblasts in Wound Tissue, Ann. Surg. 91, 641-650
- Horn, M. J., Jones, D. E., and Blum, A. E. (1944) Colorimetric Determination of Methionine in Proteins and Food, J. Biol. Chem., 166, 313-320

- Howes, E. L., Sooy, J. W., and Harvey, S. C. (1929) The Healing of Wounds as Determined by Their Tensile Strength, J. A. M. A., 92, 42-45
- Howes, E. L., Flotz, C. M., Blunt, J. W., and Ragan, C. (1950) Surgery, 28, 177
- Howes, E. L. and Harvey, S. C. (1932) The Age Factor in the Velocity of the Growth of Fibroblasts in Healing Wound Tissue, J. Exp. M., 53, 577-589
- Howes, E. L., Briggs, H., Shea, R., and Harvey, S. C. (1933) Effect of Complete and Partial Starvation on the Rate of Fibroplasia in the Healing Wound, Arch. Surg., 27, 846-858
- Hubbel, R. B., Mendel, L. B., and Wakeman, J. J. (1937) A New Salt Mixture for Use in Experimental Diets, J. Nutrition, 14, 273-285
- Hunt, A. H. (1941) Role of Vitamin C. in Wound Healing, Brit. J. Surg., 28 436-461
- Kobak, M. W., Bennett, E. P., Wissler, R. W., and Steffee, C. H. (1947) The Relation of Protein Deficiency to Experimental Wound Healing, Surg. Gyn. Obst., 85, 751-756
- Kratzer, F. H., Williams, D. E., and Jones, A. (1948) The Glycine Requirement of Young Poults, J. Nutrition, 35, 315-320
- Layton, L. L. (1950) In Vivo Sulfate Fixation by Granulation Tissue and Injured Muscle Tissue from Healing Wounds, Proc. Soc. Exp. Biol. and Med., 73, 570-572
- Lewis, R. B. (1936) The Metabolism of Sulfur, J. Biol. Chem., 110, 23-31
- Lind, J. (1772) A Treatise on the Scurvy, London
- Localio, A. S., Casale, W., and Hinton, J. W. (1948) Wound Healing; Experimental and Statistical Study, Surg. Gyn. Obst., 77, 369-375
- Localio, A. S., Morgan, M. E., and Hinton, J. W. (1948) The Biological Chemistry of Wound Healing, Surg. Gyn. Obst., 86, 582-590
- Maconshi, E. and Demetrescu, C., Acad. Rep. Populare Romane. Bul. Stunt. A. 1, 599-613 (1949)
- Madden, S. C., Anderson, F. W., Donovan, J. C. (1945) Plasma Protein Production Influenced by Amino Acid Mixtures and Lack of Essential Amino Acids, J. Exp. M., 82, 77-92

- Mariani, A., Mazzarella, O. (1950) Protein Content of the Blood and of Some Tissue of Some Surgical Patients, Boll. ist. aliment. dietal., 1, 58-70
- Marsters, N. (1939) The Determination of Sulfur in Biological Material, Biochemical J., 33(2), 1313-1323
- McCarthy, T. E. and Sullivan, M. X. (1941) A New and Highly Specific Colorimetric Test for Methionine, J. Biol. Chem., 141, 871
- Medes, G. (1939) Metabolism of Sulfur, Biochemical J., 33, 1559-1569
- Medes, G. and Floyd, N. (1942) Metabolism of Sulfur, Cysteic Acid, Biochem. J., 36, 836-844
- Melchior, J. E., and Harver, H. (1947) Studies on Protein Synthesis in Vitro, Arch. Biochem., 32, 309-315
- Melchior, J. E., Mellooy, M., and Klotz, I. M. (1948) The Synthesis of Protein by Non-Proliferating Escherichia Coli, J. Biol. Chem., 174, 81-88
- Mescon, H. and Flesch, P. (1952) J. Invest. Dermatol., 16, 261
- Miller, G. L. and de Vigneand, V. (1937) The Cystine Content of Insulin, J. Biol. Chem., 113, 101-110
- Miller, L. L. (1944) The Metabolism of DL-Methionine and L-Cystine in Dogs on a Very Low Protein Diet, J. Biol. Chem., 152, 603-611
- Morris, H. P., Rubnik, C. S., and Dunn, T. B. (1945) Technique for Studying Epithelization of Paired Skin Wounds in Rats, With Observations on the Influence of Dietary Proteins, J. Natl. Cancer Inst. 5(4), 271-282
- Mulford, D. J. and Griffith, W. H. (1942) Choline Metabolism, J. Nutrition, 23, 91-100
- Nahum, L. H., Hamilton, W. F., and Hoff, H. S. (1943) The Injury Current in the Electrocardiogram, Amer. J. Physiol., 139, 202-207
- Noble, R. L. (1950) In The Hormones, 2, 65, New York
- Paul, H. E., Paul, M. F., Taylor, J. D., and Marsters, N. W. (1948) Biochemistry of Wound Healing, Arch. Biochem., 17, 269-275
- Paveck, P. L. and Baum, H. M. (1941) The Relation of Cystine to Achromotrichia, Proc. Soc. Exp. Biol. and Med., 47, 271-272

- Penney, J. R. and Ralfour, B. M. (1949) The Effect of Vitamin C on Mucopolysaccharide Production in Wound Healing, J. Path. Bact., 61, 171-178
- Perez-Tamayo, R. and Ihnen, M. (1953) The Effect of Methionine in Experimental Wound Healing; A Morphologic Study, Am. J. Pathology, 29, 233
- Peters, R. A. (1945) The Biochemical Lesion in Thermal Burns, Brit. Med. Bull., 3, 81-88
- Peters, R. A. (1948) Vitamin C Requirement of Human Adults, Lancet, 254, 853-858
- Petrina, M. (1951) Arch. Sci. Med., 21, 285-295
- Pirani, C. L., Stepto, E. C., and Sutherland, K. J. (1951) Desoxycorticosterone Acetate and Wound Healing, Exptl. Med., 93, 217-228
- Reid, M. E. (1948) Urinary Excretion of Ascorbic Acid by Guinea Pigs with Healing Skin Wounds, Am. J. Physiol., 152, 446-454
- Rhoads, J. E., Fliegelman, W. T., and Panzer, L. E. (1942) The Mechanism of Delayed Wound Healing in the Presence of Hypoproteinemia, J. A. M. A., 118, 21-25
- Robson, H. R. and Guthrie, J. J. R. (1950) Capillary Resistance and Adrenocortical Activity, Brit. M. J., 2, 971-977
- Rogers, C. S. (1950) Amer. J. Physiol., 163, 347
- Rose, W. C. (1938) The Nutritive Significance of the Amino Acids, Physiol. Revs., 18, 109-136
- Rose, W. C. and Wood, T. R. (1941) The Synthesis of Cystine in Vivo, J. Biol. Chem., 141, 381-389
- Rose, W. C., Haines, W. J., and Johnson, J. E. (1942) Transmethylation as a Process in Man, J. Biol. Chem., 146, 683-694
- Rose, W. C. and Warner, D. T. (1943) Further Experiments on the Role of Amino Acids in Human Nutrition, J. Biol. Chem., 148, 457-469
- Rossiter, R. J. and Clark, E. J. (1944) Med. Res. Coun. Bull. War Med. 4, 256
- Sadasivan, V. (1950) Current Sci. (India), 19, 286-287

- Sahasrabudhe, M. R. and Rao, M. V. L. (1951) Effect of Vitamin B₁₂ on the Synthesis of Protein and Nucleic Acids in the Liver, Nature, Lond., 168, 605-606
- Sandblom, P., Petersen, P., and Muren, A. (1953) Determination of the Tensile Strength of the Healing Wound as a Clinical Test (Skin) Acta. Chir. Scan., 105, 252-257
- Schoenheimer, R. S., Ratner, S., and Rittenberg, D. (1939) Studies in Protein Metabolism, J. Biol. Chem., 130, 703-732
- Schoenheimer, R. S. and Rittenberg, D. (1940) The Study of Intermediary Metabolism of Animals With the Aid of Isotopes, Physiol. Revs. 20, 218-244
- Simpson, M. V., Farber, E., and Tarver, H. (1950) Studies on Ethionine J. Biol. Chem., 182, 81-89
- Simpson, M. V. and Tarver, H. (1950a) Studies on Protein Synthesis in Vitro: Further Observations on the Incorporation of Methionine into Liver Protein, Arch. Biochem., 25, 384-395
- Simpson, M. V. (1953) The Release of Labeled Amino Acids from the Proteins of Rat Liver Slices, J. Biol. Chem., 201, 143-154
- Spain, D. M., Molomut, N., and Haber, A. (1950) Am. J. Path., 26, 710
- Sylvén, B. (1941) Occurrence of High Molecular Sulfate Esters in Granulation Tissue and in Epithelial Regeneration, Acta Chir. Scand., 66, 1
- Tarver, H. and Schmidt, C. L. A. (1939) The Conversion of Methionine to Cystine; Experiments With Radioactive Sulfur, J. Biol. Chem. 130, 67-80
- Tarver, H. and Schmidt, C. L. A. (1942) Radioactive Sulfur Studies, J. Biol. Chem., 146, 69-84
- Tarver, H. and Morse, L. M. (1943) The Release of the Sulfur From the Tissues of Rats Fed Labeled Methionine, J. Biol. Chem., 173, 53-61
- Tarver, H. (1950) Advances in Biology and Medical Physics, II, 281
- Tarver, H. and Solomon, J. (1952) The Effect of Diet on the Fate of Loss of Labeled Amino Acids From Tissue Proteins, J. Biol. Chem., 195, 447-452
- Tauberhaus, M. and Amromin, G. D. (1950) The Effects of the Hypophysis,

Thyroid, Sex Steroids, and the Adrenal Cortex Upon Granulation Tissue, J. Lab. and Clin. Med., 36, 7-13

Thompson, W. P., Ravdin, I. S., and Frank, I. L. (1938) Effect of Hypoproteinemia on Wound Disruption, Arch. Surg., 36, 500-508

Treon, J. P. and Grutchfield, W. E. (1942) Rapid Turbidimetric Method for Determination of Sulfates, Indust. Engin. Chem., 14, 119-121

Van Scott, E. J. and Fleisch, P. (1954) Sulfhydryl and Disulfide in Keratinization, Science, 119, 70-71

Virtue, R. W. and Foster-Virtue, M. E. (1938) Studies on the Production of Taurocholic Acid in the Dog; Cystine Disulfoxide, Cystine, Sulfuric Acid, and Cysteic Acid, J. Biol. Chem., 127, 431-439

Walker, E. J. (1924) Physiol. (London), 59, 32

Whipple, A. O. (1940) The Lag Period in the Healing of Wounds, Ann. Surg. 112, 481-488

Whipple, G. H., Robscheit, F. S., and Miller, L. L. (1946) Blood Protein Regeneration and Interrelation, Ann. New York Acad. Sc., 47, 317-326

Williams, E. W., Mason, L. B., and Bradshaw, H. H. (1953) Factors Affecting Wound Healing, Surg. Forum, Proc. 36th Clin. Congr. Am. Coll. Surgeons, 410-417

Williamson, M. B., McCarthy, T. H., and Fromm, H. J. (1951) Relation of Protein Nutrition to the Healing of Experimental Wounds, Proc. Soc. Exp. Biol. and Med., 77, 305

Williamson, M. B., McCarthy, T. H., and Fromm, H. J. (1951a) Metabolism of Protein Nitrogen During the Healing of Experimental Wounds, Fed. Proc., 10, 270

Williamson, M. B. (1951b) Personal communication

Williamson, M. B. and Fromm, H. J. (1952) Effect of Cystine and Methionine on the Healing of Experimental Wounds, Proc. Soc. Exp. Biol. and Med., 80, 623-626

Williamson, M. B. and Fromm, H. J. (1952a) Effect of Cystine and Methionine on the Healing of Experimental Wounds, Fed. Proc., 11

Williamson, M. B. and Neumann, G. (1954) Personal communication

Winnick, T., Friedberg, F., and Greenberg, D. M. (1948) The Utilization of

Labeled Glycine in the Process of Amino Acid Incorporation by the Protein of Liver Homogenate, J. Biol. Chem., 175, 117-126

Womack, M. and Rose, W. C. (1941) The Partial Replacement of Dietary Methionine by Cystine for Purposes of Growth, J. Biol. Chem., 141, 375-379

Zittle, C. A. and O'Dell, B. A. (1941) The Determination of Cystine: The Use of Cuprous Oxide for Simultaneous Reduction and Precipitation of Cystine as the Cuprous Mercaptide, J. Biol. Chem., 139, 753

APPROVAL SHEET

The dissertation submitted by Herbert J. Fromm has been read and approved by five members of the faculty of the Stritch School of Medicine, 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 13, 1954
Date

Martin B. Williamson
Signature of Adviser