1959

Hormones and Wound Healing

Lilita Straumanis
Loyola University Chicago

HORMONES AND WOUND HEALING
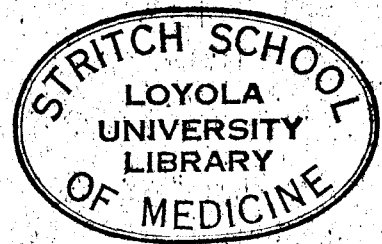
by

Lilita Straumanis

A Thesis Submitted to the Faculty of the Graduate School

of Loyola University in Partial Fulfillment of

the Requirements for the Degree of

Master of Science

February

1959

LIFE

The Author, Lilita Straumanis, was born in Riga, Latvia on August 21, 1931. She graduated from the Rolla High School in Rolla, Missouri in June, 1952. Her undergraduate training was begun at Cottey Junior College in Nevada, Missouri in 1952. The Degree of Bachelor of Arts with a major in Chemistry was awarded her in June, 1954 from the Oklahoma College for Women at Chickasha, Oklahoma. From July, 1954 to July, 1956, the author was employed as Research Technician at the University of Texas Cancer Research Hospital in Houston, Texas. In September, 1956 she was accepted as candidate for the Master of Science Degree in the Department of Biochemistry of Loyola University in Chicago.

TABLE OF CONTENTS

PREFACE

The healing of wounds involves growth processes. These processes include development and mitosis of cells, and the synthesis of tissue components. In order that cells may divide, grow and metabolize normally, either in a young growing organism or in healing wounds, the proper building materials must be available. There must be sources of energy so that these anabolic processes may go forward. The physical environment must be adequate for these reactions. Many factors influence and modify the rate of the processes connected with cell proliferation and growth. Modification of any of these factors or processes can be expected to affect growth as well as wound healing.

Changes in the normal balance of several hormonal factors in the body are known to alter the course of wound healing. The manner in which most of these substances exert their action is unknown. For instance, it is not known how a given hormone may affect the metabolic changes which accompany wound healing.

It has been shown that wound healing involves changes in sulfur metabolism. Synthesis of proteins rich in sulfur amino acids occurs in the wound tissue. There appears to be an acceleration in sulfur metabolism, and an increased retention of sulfur. (Williamson and Fromm, 1955) In this thesis

1

will be discussed the results of an investigation on the action of certain hormonal factors from wounded animals on the metabolism of normal animals.

I GENERAL ASPECTS OF WOUND HEALING

Sequence of Events in Healing

When injury occurs, the wounding object leaves behind it a mass of dead and injured cells. Blood vessels are damaged causing bleeding and incomplete circulation in the injured tissue. An accumulation of metabolic products results in the wound area, probably contributing to arteriolar and capillary dilatation and to increased capillary permeability, which in turn causes an exudate of fibrinogen containing fluids to accumulate in the injured area. The blood and the exudates soon clot on top of the wound. This is the start of the formation of the scab, which consists mostly of tissue fragments, and clotted blood and fibrinogen containing exudates. Underneath the scab the regenerative processes begin to take place.

Needham (1952) divides the sequence of events following trauma into three parts: 1) wound closure, 2) demolition and defense, and 3) healing. The formation of the scab belongs under the first heading.

The phase of "demolition" takes place during about the first three days after wounding. During this time foreign organisms and inorganic matter in the wound area are removed by ingestion or phagocytosis by polymorph leucocytes. Apart from cells actually damaged by wounding, others may die later from oxygen starvation or from the effect of noxious agents. The dead cells are partially destroyed by autolysis with their own proteolytic enzymes, par-

3

tially removed by phagocytosis, by macrophages of the tissues, and by lympho-cytes.

After defense and demolition are completed, the actual healing starts to take place. In a skin wound, capillary loops start to grow out supplying nutrients to the increasing numbers of cells which start to appear in the wound area. These are embedded in an amorphous ground substance and include the connective tissue cells, the fibroblasts and mast cells and epithelial cells. Some of these cells probably migrate from other parts of the body; others multiply by mitosis in the wound area. New collagen fibers start to be formed by the process of fibroplasia. Progressively increasing amounts of collagen appear in the wound during the first two weeks of healing. The ten-sile strength of the wound, which has been used extensively as a criterion for the rate of healing, seems to be proportional to the amount of collagen formed in the wound. This new wound tissue is known as the granulation tissue. Usually the size of the scar ultimately resulting from wounding is smaller than the size of the original wound. Contraction of the wound, which starts with the appearance of granulation tissue in the wound, accounts for this reduction in size.

Connective Tissue

Since wounds in most organs of mammals heal largely through the formation of new connective tissue (Howes, 1954), a short description of this tissue seems to be in order. Connective tissue or mesenchyme is found through-out the whole body. Its structural components are the ground substance, fibers and cells.

The ground substance consists in part of tissue fluid, derived largely from blood plasma, but in addition it has a high content of mucopolysaccharides and mucopolysaccharide-protein complexes. Among these carbohydrate components six different kinds of mucopolysaccharides have been identified (Meyer et al., 1956). The composition of these carbohydrate components in the ground substance and their degree of polymerization greatly varies in different tissues and with the age and stage of development of a particular tissue (Catchpole, 1957); (Loewi and Meyer, 1958).

Collagen is the major fibrous constituent of most types of connective tissue. It has a relatively high content of glycine and proline and accounts for practically all the hydroxyproline found in the body. (Edwards and Dunphy, 1957) Collagen is poor in the sulfur amino acids and the aromatic amino acids. The amino acid composition of collagen is shown in Table I. Collagen fibers in the native form are insoluble in water; on heating it is converted to a water soluble substance, gelatin. Under the electron microscope collagen fibers show a cross-striated appearance of a definite periodicity (640Å). (Kulonen, 1954)

The cells of the connective tissue are involved in collagen synthesis (Stearns, 1940). It is as yet unclear whether these cells elaborate collagen fibers as such, or soluble collagen precursors from which collagen fibers are precipitated in the ground substance. There is some evidence to indicate that fibers might be formed outside the fibroblast Neuberger, (1955). The mucopolysaccharides of the ground substance seem to play a part in fiber formation (Highberger et al., 1951). These carbohydrate components have their origin linked either to the mast cells (Asboe-Hansen, 1954), or possibly the fibro-

TABLE I

Amino Acid Composition of Collagen *

Amino Acid	Collagen Gm./100 gm.
Glycine	26.2
Alanine	9.5
Leucine + Isoleucine	5.6
Valine	3.4
Threonine	2.4
Methionine	0.8
Cystine	0.0
Proline	15.1
Hydroxyproline	12.83
Phenylalanine	2.5
Tyrosine	1.4
Tryptophane	0.0
Arginine	8.8
Histidine	0.8
Hydroxylysine	1.3
Lysine	4.5
Aspartic Acid	6.3
Glutamic Acid	11.3

* Boews, Elliot and Moss, 1953

blasts (Meyer, 1947).

Metabolic Events During Wound Healing

The strength of the healing wound is dependent on the formation of collagen fibers. In the healing wound, as in other growing tissues, the mature collagen fibers seem to be formed from a precursor, precollagen (Orekhovitch, 1950). There is evidence that in such tissues the activity of the connective tissue cells, the composition of ground substance and collagen formation are closely related (Dunphy and Udupa, 1955). The composition of the ground substance in growing and regenerating tissues differs from that of tissues that have ceased proliferating. Embryonic tissues and granulation tissues from healing wounds have been reported to absorb in vitro S^{35} labeled sulfate from the medium, while uninjured muscle tissue showed no such capacity (Layton, 1950. Moltke (1957) has reported an increased incorporation of S^{35} labeled sulfate in wound tissue of skin wounds as compared to skin tissue from the same animals. It has been established that practically all of the S^{35} found in the skin, after S^{35} sulfate administration, is incorporated in sulfated mucopolysaccharides (Bostrtm and Gardell, 1953).

Immediately after the start of the repair period there is a rise in the polysaccharide concentration of the wound tissue. As the collagen content increases, the mucopolysaccharides decrease (Dunphy and Udupa, 1955). During the first days of healing the mucopolysaccharide constituents of the ground substance contain more soluble components than later during the course of healing. This is true also in developing tissues from the fetal and newborn animal, and probably indicates that ground substance in growing and regenerat-

ing tissues contains mucopolysaccharide constituents which are in a less polymerized state than in mature tissues (Gersh and Catchpole, 1949; Catchpole, 1947). The relative amounts of several established mucopolysaccharides of the ground substance of embryonic skin have been found to be different from those of adult skin (Loewi and Meyer, 1958).

Along with the increase in wound strength and collagen content of the granulation tissue, there is also an increase in the concentration of the sulfur amino acids in this tissue (Williamson and Fromm, 1955). These amino acids are probably utilized for protein synthesis in the wound tissue. During the first few days of healing there seem to be an accumulation of proteins which are relatively rich in methionine and low in cystine. Later, proteins having a higher percentage of cystine are synthesized.

Since wound healing involves increased synthesis of new proteins, the protein metabolism of an injured animal could be expected to be different from that of a normal animal. After wounding there is an increased excretion of nitrogen in the urine, and a negative nitrogen balance. However, the ratio of sulfur to nitrogen excreted is not such as one might expect from the breakdown of tissue protein. There appears to be a net retention of sulfur (Williamson et al., 1951). This seems to indicate that after wounding, although body proteins are broken down at an increased rate, the sulfur amino acids are not excreted to the same extent as are the other amino acids. It seems that during repair of wounds body proteins are broken down at an increased rate to provide a larger pool of the sulfur amino acids which can then be used in the healing process, probably for the synthesis of the sulfur amino acid-rich proteins mentioned before. The remaining amino acids then are left

in such a ratio that they cannot be utilized for protein synthesis.

It has been shown that if wounded animals are fed a high protein diet, their wounds heal faster than wounds in animals fed a low-protein diet. However, there seems to be no relation between the rate of healing of wounds and nitrogen intake and retention. Healing seems to be influenced by the presence of the sulfur amino acids in the diet. The addition of methionine or cystine to a low-protein diet causes an increase in the rate of healing (Williamson et al., 1952, 1953, 1954). Other amino acids, essential and non-essential, do not seem to have this effect (Morris et al., 1945).

The sulfur of methionine can be irreversibly converted to cystine. Since cystine supplementation also has a beneficial effect on the rate of healing, it seems highly probable that this amino acid is the limiting factor in the processes involving amino acid metabolism and wound healing (Williamson et al., 1954).

Studies by Williamson and Fromm (1955) with S^{35} labeled methionine and cystine indicate that during healing of wounds, there is an increased rate of sulfur metabolism. There is a rapid incorporation of the label into the wound tissue. The uptake and loss of methionine S^{35} is greater in skin and muscle tissue in wounded rats than in uninjured animals. The total content of cystine of liver, skin, and muscle does not change much after wounding. However, after S^{35} cystine administration the label is lost more quickly from these tissues in wounded than unwounded animals, indicating that there is a more rapid turnover of this amino acid after wounding.

The most marked difference between wounded and unwounded animals in the S^{35} content of tissues occurs in the liver. There is a significant de-

crease in the S^{35} cystine and methionine content in the liver after wounding. There is also a decrease in the total liver methionine after wounding. These findings suggest that the liver makes a contribution of methionine to the wound. There also have been data reported which indicate that muscle methionine is mobilized to the liver after wounding (Fromm and Nordlie, 1956).

In the body, methionine is converted into cystine and this amino acid then gives rise to the inorganic sulfate which is excreted in the urine or incorporated in the mucopolysaccharides. Wounded animals excrete larger amounts of sulfate S^{35} than do unwounded rats after the administration of S^{35} labeled methionine or cystine to wounded and unwounded rats. This suggests an increased catabolism of the sulfur containing amino acids after injury (Williamson and Fromm, 1955).

Methionine is the first essential amino acid whose deficiency during protein depletion is reflected in growth and wound healing. Administration of methionine to wounded, protein depleted rats increases the collagen content in the wounds of these animals, and thus also the tensile strength of the wounds (Udupa et al., 1956). In protein depleted animals there appears to be a defect in the sulfation of mucopolysaccharides in the granulation tissue (Perez-Tamayo and Ihnen, 1953). Dietary methionine seems to restore this process towards normal (Edwards and Dunphy, 1957). The mechanism of the role of methionine during the healing process is not known.

Dunphy et al. (1955) have postulated several mechanisms to explain the action of methionine in restoring healing towards the normal in protein depleted animals. It is known that a portion of the sulfur amino acids utilized in the healing of wounds in rats maintained on a protein free,

methionine supplemented diet, are derived from the breakdown and utilisation of liver and muscle proteins (Williamson and Fromm, 1955). Methionine increases the biological value of dietary protein and increases the nitrogen balance in rats (Forbes, 1954). Similarily this amino acid might exert an influence in increasing the efficiency of the metabolism of the proteins derived from other body tissues for use in the repair process.

It has been shown that chick fibroblasts cultured on a synthetic culture medium require the sulfur amino acids for survival. Moderate culture survival occurs in the absence of methionine, provided that adequate cystine is present in the medium. For maximum survival, methionine is necessary in addition to cystine. (Morgan and Martin, 1955). Dunphy et al. have considered the possibility that methionine might act directly upon the proliferation of fibroblasts.

Edwards and Dunphy (1957) postulate that an important mechanism of action of methionine in healing in protein starved animals might be connected with the metabolism of vitamin C and the adrenal steroids, this effect residing in an altered ration of adrenal steroids excreted. Protein starvation has been found by these workers to depress ascorbic acid metabolism in the rat. The administration of methionine tends to restore the metabolism of ascorbic acid in the tissues of these animals towards normal. The changes in adrenal ascorbic acid seem to be accompanied by changes in the metabolism of adrenocortical hormones. Adrenalectomy appears to have a similar normalising effect as methionine supplementation on healing in protein starved animals.

II HORMONAL INFLUENCE ON WOUND HEALING

Disturbances in the normal hormonal balance in the body often result in alterations in the composition of connective tissue. This has been shown to be the case with growing and developing connective tissue as well as in healing wounds. (Asboe-Hansen, 1950, 1954; Castor and Baker, 1950; Holden and Adams, 1957; Edwards and Dunphy, 1957; Howes, 1954; Moltke, 1957; Ragan et al., 1949; Taubenhaus and Amromin, 1950) One might suppose that in some cases these alterations would be a result of general changes or disturbances in the metabolism of the organism as a whole. For instance, disturbances in carbohydrate, protein, or mineral metabolism might be reflected in reactions involved in the maintainence, growth or regeneration of the elements of the mesenchymal tissues. However, there are also indications that hormones exert a direct effect on connective tissue; their action being reflected on all three components of this tissue cells, fibers, and ground substance. Since the intercellular substances are usually derived from and dependent upon the cellular elements, hormonal influences on cells probably have effects on the structure of the other two components. Conversely, because the process of wound healing involves anabolic and growth phenomena, it requires alterations in the metabolic events in the organism as a whole, in addition to specific events in the injured tissue. The changes in nitrogen and sulfur metabolism in the organism during wound healing were briefly described before. These altered metabolic reactions, and the

12

growth processes in the injured tissues, could conceivably be dependent on changes in the secretion of various hormones, or an altered sensitivity to hormones by target tissues, these effects being originally brought about by the stimulus of wounding.

It is evident that there is an interdependence among the various hormones as they influence the metabolism of the body as a whole or as they affect specifically the composition of mesenchymal tissues. This probably can also be extended to the process of wound healing.

Thyroid Hormone and Thyrotrophin

Early reports concerning the effect of thyroid hormone on wound healing seem to indicate that this hormone increases the rate of healing (Arey, 1936; Needham, 1952). However, later work shows that thyroxine changes the morphological composition of the wound tissue and that it delays the increase in tensile strength of granulation tissue (Moltke, 1955).

The primary effect of the administration of thyroxine to normal animals is an increased oxygen consumption by these animals. Administration of thyroxine to normal animals leads to alterations in the dermal connective tissue, which include a reduction in the content of mucopolysaccharides and the number of mast cells in this tissue (Asboe-Hansen, 1950).

The release of the thyroid hormone from the thyroid gland is stimulated by the thyrotrophic hormone of the anterior pituitary. The release of this hormone, in turn, is regulated by the amount of thyroid hormone in circulation. In addition to stimulating the activity of the thyroid gland, there is evidence that the thyrotrophic hormone also exerts a direct effect on connec-

tive tissue by stimulating an increased accumulation of mucopolysaccharides. The changes observed after thyrotrophic hormone stimulation, although being most marked in the retrobulbar connective tissue of the eye, are also found in peripheral muscles, perirenal, auxillary and peritesticular regions. Thyroxine inhibits the exophthalmic effect (Smelser, 1938; Asboe-Hansen and Iversen, 1952).

Histological study of granulation tissue surrounding turpentine abscesses by Taubenhaus and Amromin (1950) indicated that thyroidectomy results in the formation of a larger amount of intercellular substance in the granulation tissue than usual. Thyroxine seems to stimulate the formation of granulation tissue in thyroidectomized rats. These workers also report that thyroxine does not alter the structure of granulation tissue in intact rats.

Moltke (1955), using the increase in tensile strength as a measure of the healing rate, showed that thyroxine delays healing in skin wounds in guinea pigs. This effect was produced by the injection of thyroxine both in thyroidectomized and in intact animals. Throtrophic hormone was shown to have no effect on the rate of healing in thyroidectomized guinea pigs, but to inhibit healing in intact animals. The lack of the thyroid hormone did not seem to affect healing since thyroidectomized and intact controls showed similar rates of healing. Studying the incorporation of S^{35} labeled sulfate into wound tissue, Moltke (1957) found that thyroxine inhibited the fixation of S^{35} sulfate into wounds, but not into intact skin in wounded guinea pigs.

A deficiency of vitamin C is known to delay the healing of wounds, ascorbic acid having a role in the formation of high-molecular weight acid mucopolysaccharides and of collagen fibrils (Robertson and Hinds, 1956;

Danielli, Fell and Kodicek, 1945). Moltke (1956) investigated the possibility that the inhibition of thyroxine upon wound healing might be due to an antagonism of thyroxine to ascorbic acid. He found that the inhibiting effects of thyroxine and scurvy were not interdependent, the effects of thyroxine upon the rate of increase in tnesile strength of wounds in guinea pigs not being affected by the supplementation of ascorbic acid.

Adrenal Cortical Hormones

The adrenocorticotrophic hormone, through its stimulation of the adrenal gland, is known to affect the composition of connective tissues. Relatively large doses of ACTH or cortisone have the effect of diminishing the number of both fibroblasts (Castor and Baker, 1950; Holden and Adams, 1957) and of mast cells (Asboe-Hansen, 1952; Zachariae and Moltke, 1954) in the tissues of many species of animals. The morphological appearance of the cytoplasm of the mast cells is altered in that there is a loss of granules, which are rich in sulfated mucopolysaccharides. The formation of components of the ground substance is inhibited. Asboe-Hansen (1954) found that mast cells in tumor connective tissue from cortisone treated mice had a lower uptake of S^{35} labeled sulfate than did the mast cells from untreated mice. Further data by Layton (1951) and Rice (1956) indicate that cortisone inhibits the formation of sulfated mucopolysaccharides. Schiller and Dorfman (1957) have reported that not only the uptake of S^{35} labeled sulfate in chondroitin sulfuric acid, but also the incorporation of the lable from C^{14} acetate in hyaluronic acid in the skin of rats is decreased by the administration of cortisone. Half-life determinations showed also a decrease in the turnover of chondroitin sulfuric

acid and hyaluronic acid.

Collagen fiber formation is also inhibited upon cortisone treatment (Gerarde and Jones, 1953; Castor and Baker, 1950). The possibility has been considered that the primary effect of cortisone is on the connective tissue cells, which are the source of the other important connective tissue components, the mucopolysaccharides and the collagen fibers (Taubenhaus and Amromin, 1950; Taubenhaus, 1953; Spain and Molomut, 1950).

The effects of the adrenal steroids on the cellular components of granulation tissue are similar to the effects of these steroids on the cells of connective tissue, a smaller number of mast cells and fibroblasts being found in granulation tissue of cortisone treated animals than in untreated controls. The fibroblasts in the granulation tissue of cortisone treated animals are smaller in size (Ragen et al., 1949; Taubenhaus and Amromin, 1950) and the mast cells become degranulated (Asboe-Hansen, 1954).

The data concerning the effects on healing by the adrenal cortical steroids, and especially the ACTH, have been quite inconsistent. This probably is due to the fact that a high dosage of these compounds is required for most animals, the sensitivity to the corticoids varying with the species and the physical state of the animals, and with the type of injury. ACTH probably exerts its effect through a stimulation of the adrenal cortex to produce hydro-cortisone. Even small doses of cortisone interfere with healing if the animal is malnourished (Findlay and Howes, 1952).

Administration of cortisone and hydrocortisone is known to produce a negative nitrogen balance. However, this action of the adrenal cortical steroids is not the sole cause of the effects on wound healing, for it has been

found that local application of cortisone or hydrocortisone to experimental wounds interferes with the healing process (Shapiro, Taylor, and Taubenhaus, 1951). Morphological changes appearing in granulation tissue which has been locally treated with these steroids correspond to those in wounds during systemic cortisone administration (Zacharias and Moltke, 1954).

Desoxycorticosterone, which has been classed with respect to its actions on connective tissues as an "inflammatory" hormone, as contrasted to cortisone and hydrocortisone as being "anti-inflammatory" hormones (Selye, 1954), exerts effects on granulation tissue which appear to be opposed to the action of the latter steroids. Taubenhaus and Amromin (1949) have reported that prolonged treatment with desoxycorticosterone acetate resulted in stimulated formation of granulation tissue around turpentine induced abscesses in rats, the amount of the tissue being increased (Taubenhaus, 1949; Pirani et al., 1951). Desoxycorticosterone acetate may counteract the inhibitory effect of cortisone on granulation tissue (Taubenhaus et al., 1952).

Growth Hormones

Hypophysectomy in all speicies observed is known to cause retardation of growth. The amministraiton of crude pituitary extracts or purified growth hormone to either hypophysectomized or intact animals of many species results in an increased rate of growth. The increase in body weight produced by growth hormone is reflected in an enlargement of the skeletal system and of the other tissues of the body, there being an increased retention of nitrogen and an increase in the quantity of protein in the body.

Loss of the anterior pituitary leads to a tendency to hypoglycemia and

loss of hepatic and muscle glycogen on fasting. There is an increased sensitivity to insulin and in increased rate of utilisation of carbohydrate. The utilization of depot fat seems to be decreased. Purified preparations of growth hormone can reverse all these effects and can exert effects of an opposite type in normal animals. It has been shown that growth hormone preparations restore the decreased concentration of liver RNA observed in hypophysectomized rats, the DNA being little affected (Li and Evans, 1948).

Wound healing has been found to be markedly reduced in hypopysectomized rats. The formation of granulation tissue is very scanty. Taubenhaus and Amromin (1950) observed that administration of growth hormone to hypophysectomized rats causes wounds to heal normally. Depending upon the amount of hormone used, the amount of granulation tissue in these animals may even be increased above normal.

If normal rats are given growth hormone in moderate amounts, healing is stimulated. However, excessive amounts of growth hormone have been found to inhibit wound healing. The morphological appearance of this growth hormone-stimulated granulation tissue is changed in that there are increased numbers of fibroblasts, these cells being large, but otherwise of a normal contour and arrangement. The formation of collagen fibers is increased, but the ground substance does not seem to differ from that of wounds of untreated animals (Taubenhaus and Amromin, 1950).

Adrenalectomy has been reported not to affect wound healing, however, the presence of the adrenal cortex seems to be necessary for stimulation of granulation tissue formation by growth hormone (Taubenhaus et al., 1952).

"Wound Hormones"

The fact that cell proliferation is initiated in an injured tissue, has caused many theories to be put forward as to the stimulus to the healing process. The theory which has caused the most experimental investigation was the "wound hormones" theory. (Arey, 1939; Teir, 1950; Sandblom, 1949; Auerbach, 1952) It was thought that injured cells might release factors which would have an effect on mitosis. Early work on the factors influencing wound healing had consisted mainly of the direct application of various tissues, tissue extracts and other substances to the wounds. Extracts of crushed tissues, which were thought to contain growth promoting factors and to be of importance in the healing of wounds, have been used in vitro to study growth in tissue cultures (Arey, 1939). Absence of standardisation in the products administered and differences in the way the experiments had been conducted, resulted in contradictory and equivocal results.

Later, studies were reported concerning the effects of growth promoting tissue extracts on healing wounds in experimental animals. These seemed to indicate that there is a basis for the supposed existence of growth promoting substances in tissue extracts (Cook and Fardon, 1942; Auerbach, 1945; Teir et al., 1951).

Teir (1950) showed that skin has a growth promoting factor which is active in the living organism. With injections of skin extracts from newborn rats a stimulation of mitotic cell division in the epithelium of the skin of young rats was obtained. Suspensions of mashed stomach, liver, and thymus tissues also showed mitosis-stimulating action on homologous tissue. Teir

further reported that these mitosis promoting extracts also influenced wound healing of skin wounds in rats, there being an increased rate of epithelialization (Teir et al., 1951; Teir, 1956).

Sandblom (1949), using tensile strength as a measure of healing, found that when two wounds were made successively in the skin of rabbits, the second wound healed faster than the first one. This finding may suggest that the stimulus of wounding releases a healing promoting factor into the circulation.

Auerbach (1952), on the other hand, observed that if the last phase of the healing process of a first skin wound in rats was made to coincide with the phase of active proliferation of the granulation tissue in a second wound in the same animal, there was an inhibition in the rate of healing of the second wound during a limited time period, suggesting the existence of an inhibiting factor.

Menkin (1956), repeatedly injecting a diffusible portion of inflammatory exudates in the skin or ear of rabbits, found proliferative response in the epithelium and the cartilage at the site of injection, while injections of plasma caused no such reaction.

III EXPERIMENTAL METHODS

In the experiments to be described, the Sprague-Dawley strain of albino rats was used. To insure uniformity and to eliminate the effects of the animal's inherent hormonal systems, only adult female rats were used. The weights of these animals at the start of the experiments ranged from 200 to 240 grams, and averaged 220 gms.

A synthetic basal diet, containing all the dietary substances known to be necessary for the maintainence of the albino rat, was used. In these experiments, however, the diet was deficient or completely lacking in protein (0-2% casein). The composition of the basal protein-free diet is shown in Table II. When the diet contained protein, it was substituted for an equivalent weight of sucrose.

The animals were kept in individual metabolism cages, and each was given 8 grams of the diet daily; this amount of food is always completely consumed at the end of 24 hours. Distilled water was given "ad libitum". To let the animals get acclimated to the synthetic diet, the feeding schedule was started three days before other experimental procedures.

Wounding Procedure

The rats were anesthesized with a subcutaneous injection of sodium Nembutal (2 mg./100 gm. rat), followed by a small amount of ethyl ether which

TABLE II

Composition of Protein-Free Diet

	Gm./100 gm. Diet
Lard	10
Corn Oil	2
Sucrose	83
Salt Mixture	5

	mg./100 gm. Diet
Thiamine Hydrochloride	1
Riboflavin	1
Pyridoxine Hydrochloride	1
Calcium Pantothenate	4
Inositol	5
P-Amino Benzoic Acid	5
Nicotine Acid Amide	15
2-Methyl Naphtaquinone	0.5
Choline Chloride	25
Vitamin A (from oleum percomorphum)	1500 IU
Vitamin	210 IU

was administered by keeping the animal's nose close to a piece of absorbent cotton soaked with ether.

The hair in the region of the back of the neck and shoulder blades was carefully cut down to the skin with scissors. A circle, 4 cm. in diameter, was traced in this area using a pen. The skin was then excised down to the muscle over the area outlined. The wounds were allowed to heal without further topical disturbance.

When at the termination of the experiment, it was desired to obtain skin and wound tissue samples, the animals were anesthetized as previously described. To obtain skin tissues, the hair on the back of the animal was clipped with scissors and then shaved with a razor. Some skin was excised, and blotted to remove any blood, and weighed wet on a Roller-Smith balance. The scab was removed from the wound and the wound tissue was excised, blotted and weighed. The tissues were placed into labeled vials and kept frozen until analyses could be carried out. Then they were dissolved by heating with 2 ml. of 0.5 N NaOH, made up to 50 ml. with distilled water, and aliquots were measured out for nitrogen, total sulfur, and S^{35} determinations.

Collection of Urine Samples

At the start of the experiments the metabolism cages were washed thoroughly with hot water and a detergent. To collect urine samples, the cages were washed down with distilled water, the washings being added to the urine already collected in a beaker. The samples were then made up to volume in 100 ml. volumetric flasks, filtered, and placed in urine bottles. A few drops of toluene were added to each sample to serve as a preservative, and the

tightly stoppered bottles were stored in a refrigerator at 5°C until analyses could be made.

Preparation of Plasma Samples

In order to study the effects of any factors released in the blood stream after wounding, it was desired to obtain plasma samples from wounded and unwounded animals. The groups of wounded and normal rats, being kept for this purpose, were given a stock diet. Wounded animals were kept in individual cages. Each day 1 mg./rat of sodium nembutal was given subcutaneously, and one to three ml. of blood was obtained from each animal by heart puncture. A small amount of heparin was used in the syringe to prevent clotting. The collection of blood was started on the day following wounding and was continued for four to six days.

The plasma was immediately separated from the cells by centrifugation, and dried from the frozen state. The dry material was placed in weighing bottles and stored in a desiccator in the refrigerator at 5°C.

Analysis for Nitrogen

The micro-Kjeldahl method was used for the determination of total nitrogen in tissue and urine samples. An aliquot of urine, or tissue hydrolyzate was measured out into an 8" test tube. One ml. of concentrated sulfuric acid was added to the tube and the mixture was digested on a sand bath for two hours. Then a few drops of 30% hydrogen peroxide were added, and the digestion continued ror another thirty minutes. Nitrogen in the form of amines or amides is converted by this procedure into ammonium sulfate.

The digested material was then transferred to a micro-Kjeldahl distillation apparatus, and made alkaline by the addition of 15N sodium hydroxide. The liberated ammonia was distilled into a measured volume of standard sulfuric acid solution. The amount of ammonia distilled into the acid was estimated by titrating the remaining sulfuric acid with a standard sodium hydroxide solution. Methyl red was used as an indicator. From the number of milliequivalents of acid and base use, the amount of nitrogen in the aliquot was estimated.

Data on recovery of nitrogen from sulfuric acid digests of glutamic acid are shown in Table III.

Total Sulfur

One ml. of concentrated nitric acid was added to an aliquot of urine, or tissue hydrolyquate and this solution was then slowly heated in an 8" test tube on a sand bath until all water had evaporated. Then the temperature of the sand bath was raised and the material in the tube was digested until the contents of the tube were almost colorless, most organic matter being oxidized. To insure the oxidation of sulfur to sulfate, a few drops of 30% hydrogen peroxide were added to the hot tube. This treatment was repeated several times, in between digesting the sample with a small amount of nitric acid. Finally all the acid was allowed to evaporate and the dry contents of the tube were analyzed for sulfate. Duplicate or triplicate samples were always prepared from each urine or tissue specimen.

The method of Toennies and Bakay (1953) was used for the determination of sulfate. This is a turbidimetric method, and depends upon the production of a suspension of barium sulfate.

TABLE III

The Recovery of Nitrogen from Glutamic Acid

Digests

Glutamic Acid	Nitrogen in Glutamic Acid	Nitrogen Found	Error
4.93 mg.	0.470 mg.	0.46 mg.	2%
14.79 mg.	1.41 mg.	1.47 mg.	4%
15.72 mg.	1.88 mg.	1.90 mg.	1%

The Recovery of Sulfate from Urine Digests
(Added to Urine as Na_2SO_4; Calculated as Sulfur)

Urine Digest	Sulfur Found	Sulfur Recovered	Error
5 ml. urine	0.18 mg.	---	---
5 ml. urine 0.15 mg S	0.34 mg.	0.16 mg.	6%
5 ml. urine 0.30 mg. S	0.49 mg.	0.31 mg.	3%
5 ml. urine 0.45 mg. S	0.61 mg.	0.43 mg.	2%

The following reagents were prepared:

1. Reagent I. To 190 ml. of 95% ethyl alcohol and 220 ml. of propylene glycol in a volumetric flask, 500 ml. of distilled water were added. Then 9.4 ml. of concentrated nitric acid were added and the contents of the flask were made up to volume with distilled water.

2. Reagent II. This reagent contained 15 ml. of 1.34 M barium chloride solution per liter of solution, otherwise having the same composition as Reagent I.

3. Standard. A weighed amount of sodium sulfate was dissolved in a portion of Reagent I to produce a solution containing 0.10 to 0.15 mg. of sulfur per ml. of solution.

To the test tube containing the digested urine or tissue sample 5.0 ml. of Reagent I were added. The tube was shaken until all of the residue had dissolved. Then 5.0 ml. of Reagent II were added from a fast-flowing pipette, the tube was shaken and after 5 minutes the degree of turbidity was read in a Klett-Summerson spectrophotometer (using the blue filter).

A standard curve was prepared by measuring out various aliquots of the standard solution, making these up to 5.0 ml. with Reagent I, adding Reagent II, and reading in the spectrophotometer. A standard curve is shown in Fig. 1. The amount of sulfur in the digested sample was estimated by reading from this curve. A curve, containing five to ten points, was prepared 48 hours within the time that sulfur determinations were run.

Data on recovery of sulfate, after known amounts of sodium sulfate were added to aliquots of urine and digested, are shown in Table III.
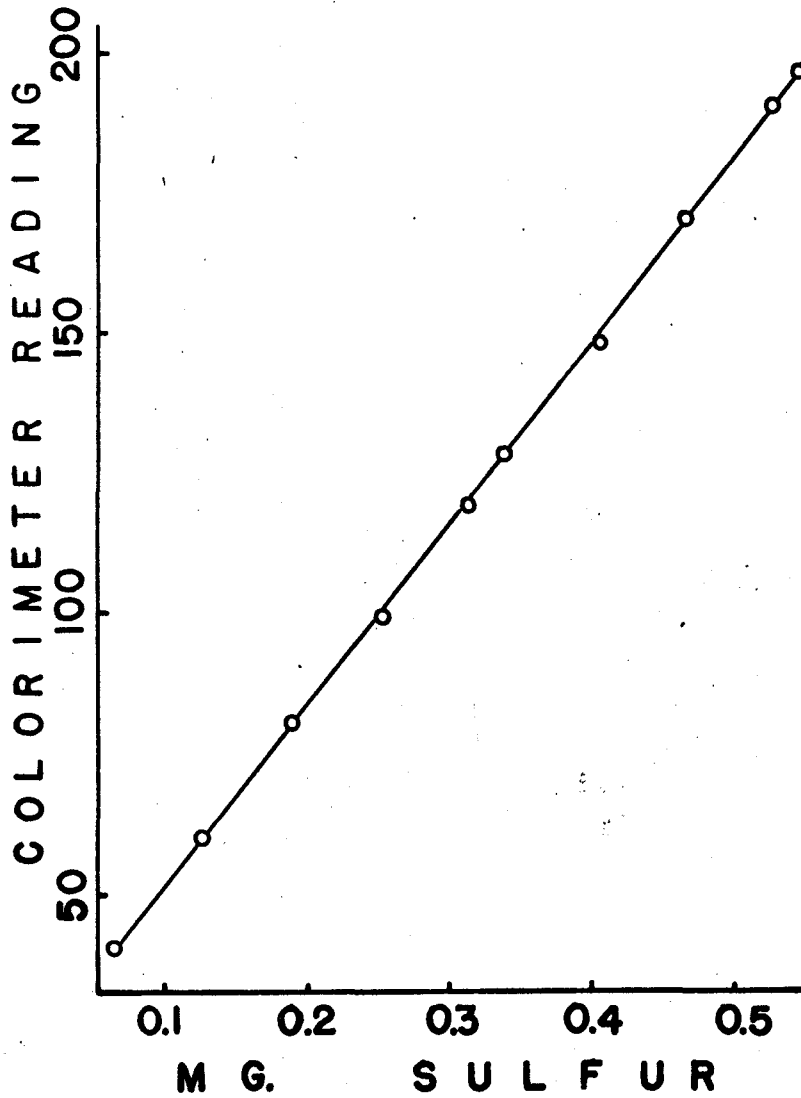
Fig. I

A Standard curve used for the estimation of sulfur

Determination of S^{35}

Duplicate aliquots of the specimen to be analyzed for total S^{35} were digested in an identical manner as was described for the determination of total sulfur. To the residue in the digestion tube was added enough sodium sulfate to make 62 mg. of barium sulfate upon addition of barium chloride. The barium sulfate precipitate was digested for 20 minutes in a boiling water bath and allowed to cool overnight before filtration.

The barium sulfate precipitate was then filtered onto a Whatman #50 filter paper 2.4 cm. in diameter using suction. A special Tracerlab filtering funnel designed for this purpose was used. After filtering was completed, the sides of the funnel and the precipitate were washed twice with acetone. Suction was continued until the precipitate was dry. Then the filter paper was placed into a stainless steel planchet.

A Tracerlab Autoscaler with an SC-16 Windowless Flow Counter was used to determine the activity of the radioactive samples. Using a standard C^{14} sample, the operating voltage of the counter was found to be 1250 volts. Background readings were made each day that samples were analyzed for S^{35} activity. Each planchet was counted two to three times, 1024 to 4096 counts being made.

The amount of 62 mg. of barium sulfate was used for each sample, since this weight of carrier had been found to produce a sample of infinite thickness. A self-absorption curve for S^{35} sulfate with barium sulfate carrier is shown in Fig. II. This curve was obtained by adding various amounts of sodium sulfate to aliquots of S^{35} labeled cystine, and then treating these as already described for S^{35} determinations.
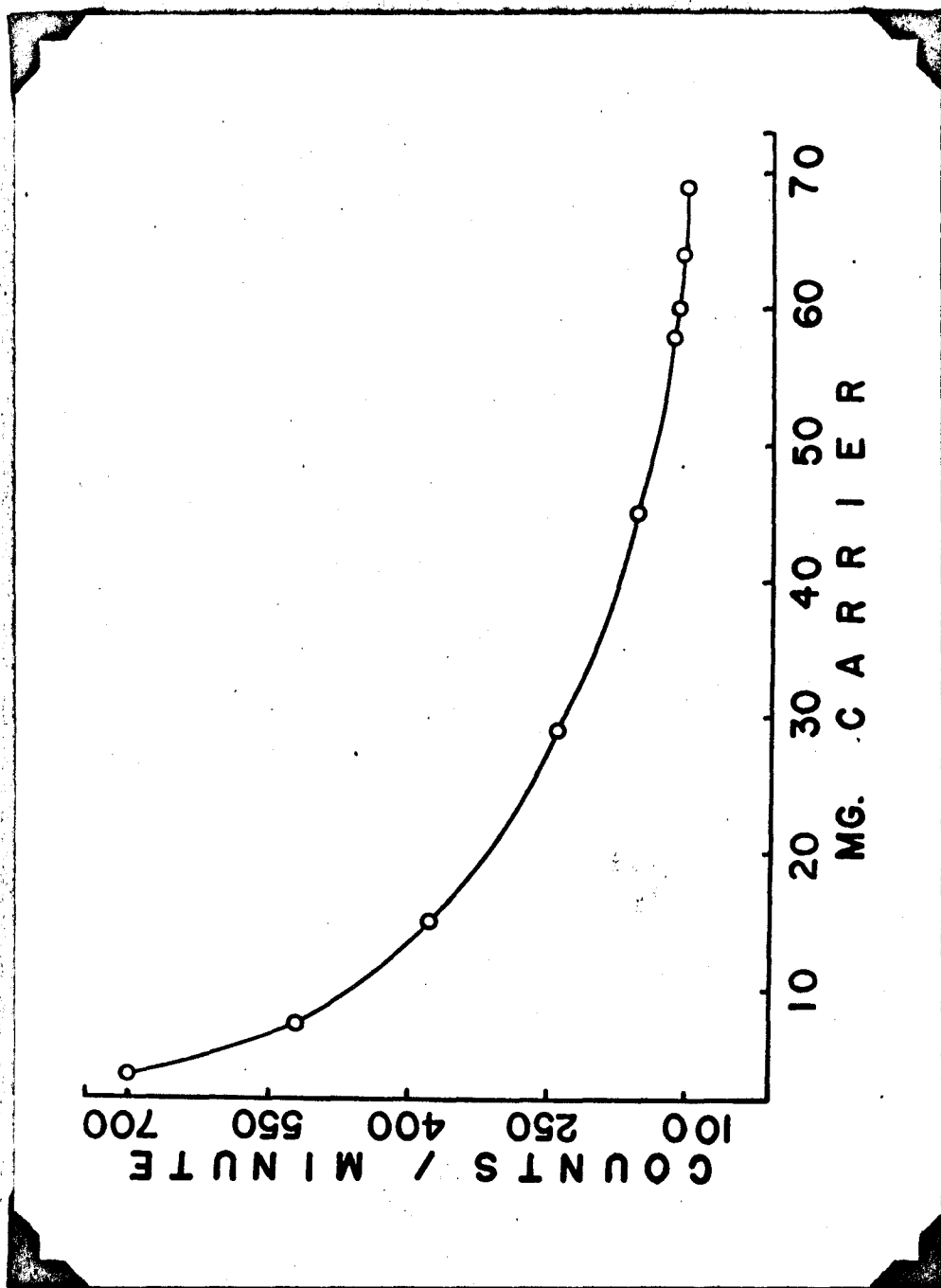
Fig. II

Self-absorption curve for S^{35}-Barium sulfate

IV RESULTS AND DISCUSSION

"Wound Factors" in Plasma

It has been reported by many workers that injured cells produce or cause the production of various factors which have the ability to alter the rate of wound healing (Aray, 1936; Auerbach, 1945; Tair et al., 1951; Mankin, 1956). It could be expected that these factors would appear in the blood as, indeed, results reported by Sandblom (1949) and Auerbach (1952) seem to indicate. It could well be that the metabolic changes observed after wounding, which include a negative nitrogen balance and retention of sulfur (Williamson and Fromm, 1954), could be brought about or at least influenced, by these hormonal factors.

To test this hypothesis, the following experiment was carried out. Three groups of eight animals were acclimated to a protein-free diet for three days. After the acclimatisation period, each animal in Group I was given an intraperitoneal injection of a solution containing 50 mg. of dried plasma, which had been previously obtained from wounded rats. The animals in Group II were given plasma from unwounded rats, and those in Group III obtained injections of physiological saline. Each rat was also injected with a solution containing 5×10^6 c/m of S^{35} labeled L-cystine. The administration of plasma and saline solution was continued for ten days, injections being made twice

daily. Thus, each rat in Groups I and II was given the equivalent of 100 mg. of dried plasma per day. During this time 24-hour urine samples were collected and analyzed for nitrogen, total sulfur, and S35. Since these animals were not fed any protein, the nitrogen and sulfur values obtained in Group III can be considered to approximately represent nitrogen and sulfur balance. The feces were not analyzed. Normally, about 90% of these elements excreted by the body are contained in the urine (Beach et al., 1942).

The data on the excretion of nitrogen by these three groups of animals are presented in Table IV and Fig. III. It can be seen that while there is no significant difference in the excretion of nitrogen between the animals receiving "wounded plasma and those receiving physiological saline, the rats which were injected "normal" plasma excreted appreciably more nitrogen than the other two groups.

Table IV and Fig. IV show data on the excretion of sulfur. It appears that the animals which were injected plasma from unwounded rats and those which did not receive any plasma, excreted similar amounts of sulfur. The group of rats which was given plasma from wounded animals, excreted less sulfur than the other two groups.

From these data it appears that the animals in Group II have responded to the administration of plasma by an increased excretion of nitrogen. This probably is due to the administration of the plasma proteins. Part of the effect might be produced by the hormonal composition of the plasma. The fact that the donor animals were bled repeatedly for several days, could conceivably have produced a condition of stress. Stressful stimuli are known to increase the concentration of ACTH in the blood (Sayers, 1957).

TABLE IV

33

Excretion of Nitrogen and Total Sulfur

(mg./rat/day)

Day*	Nitrogen Group I	Group II	Group III	Total Sulfur Group I	Group II	Group III
1	40.7	47.2	43.2	3.4	3.8	3.8
2	44.6±1.6	47.8±2.4	44.4±2.7	3.3±0.30	3.9±0.14	3.0±0.28
3	34.4±1.6	41.1±1.9	36.7±2.9	3.0±0.18	4.3±0.12	4.1±0.27
4	42.0±1.7	48.1±3.6	39.6±3.1	2.5±0.15	3.6±0.20	4.1±0.22
5	32.3±2.3	40.4±2.5	28.0±1.1	3.2±0.17	3.4±0.23	3.5±0.10
6	27.1±1.6	33.1±3.0	26.5±1.4	2.8±0.27	3.2±0.31	3.5±0.17
7	25.2±0.3	29.2±0.2	22.5±1.5	2.5±0.18	2.6±0.14	3.0±0.17
8	26.4±1.1	27.3±1.6	26.6±0.2	2.3±0.25	2.9±0.14	2.8±0.17
9	23.9±1.7	28.0±1.9	25.2±1.6	2.6±0.17	3.2±0.19	3.3±0.28
10	21.2±1.5	26.0±1.1	26.2±1.3	2.5±0.26	2.7±0.24	2.7±0.25

* Injection of plasma started on first day

Group I — wounded plasma
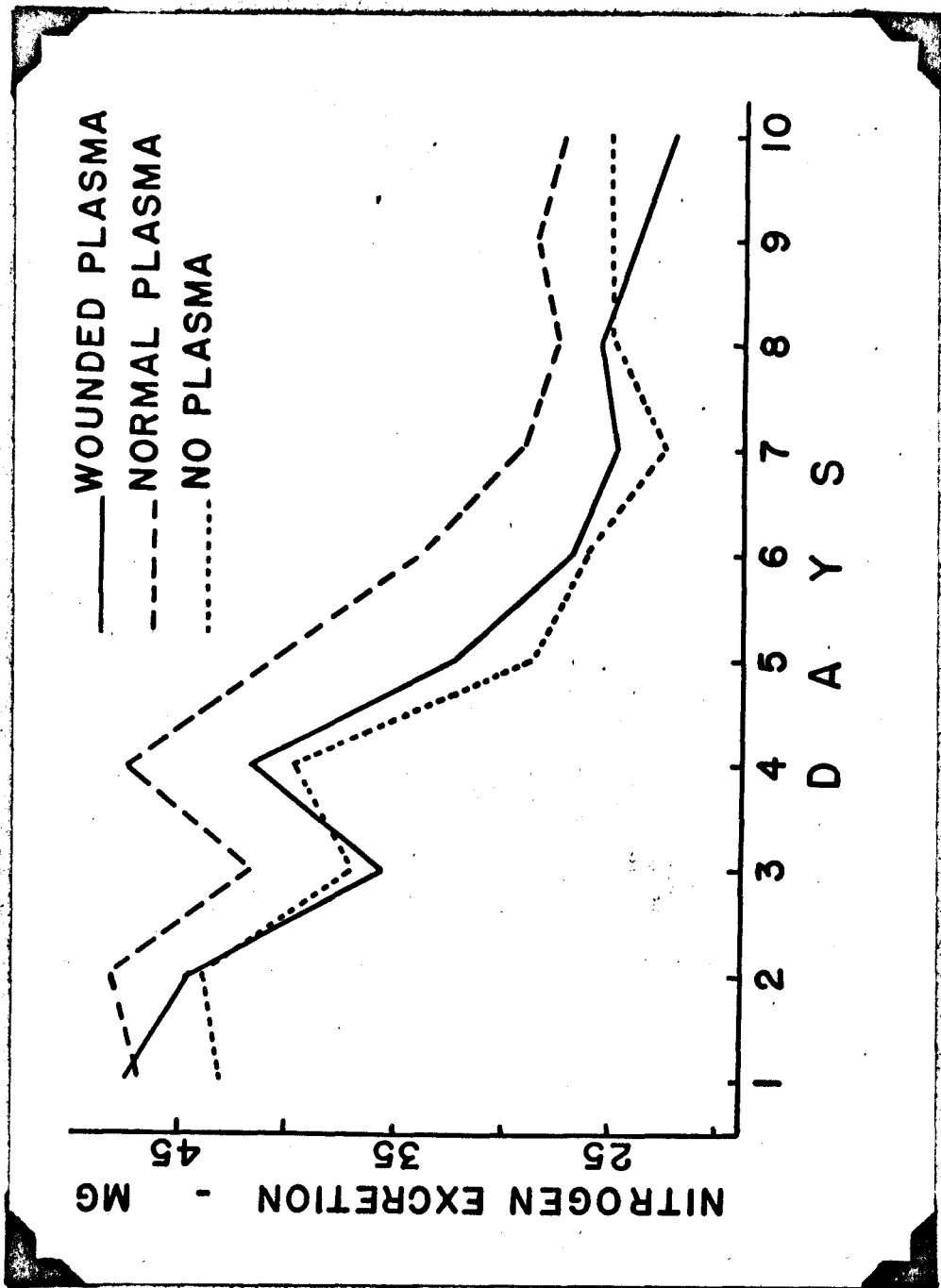
Group II — normal plasma

Group III — no plasma

34

Fig. III

Mg. Nitrogen excreted (mg./rat/day)
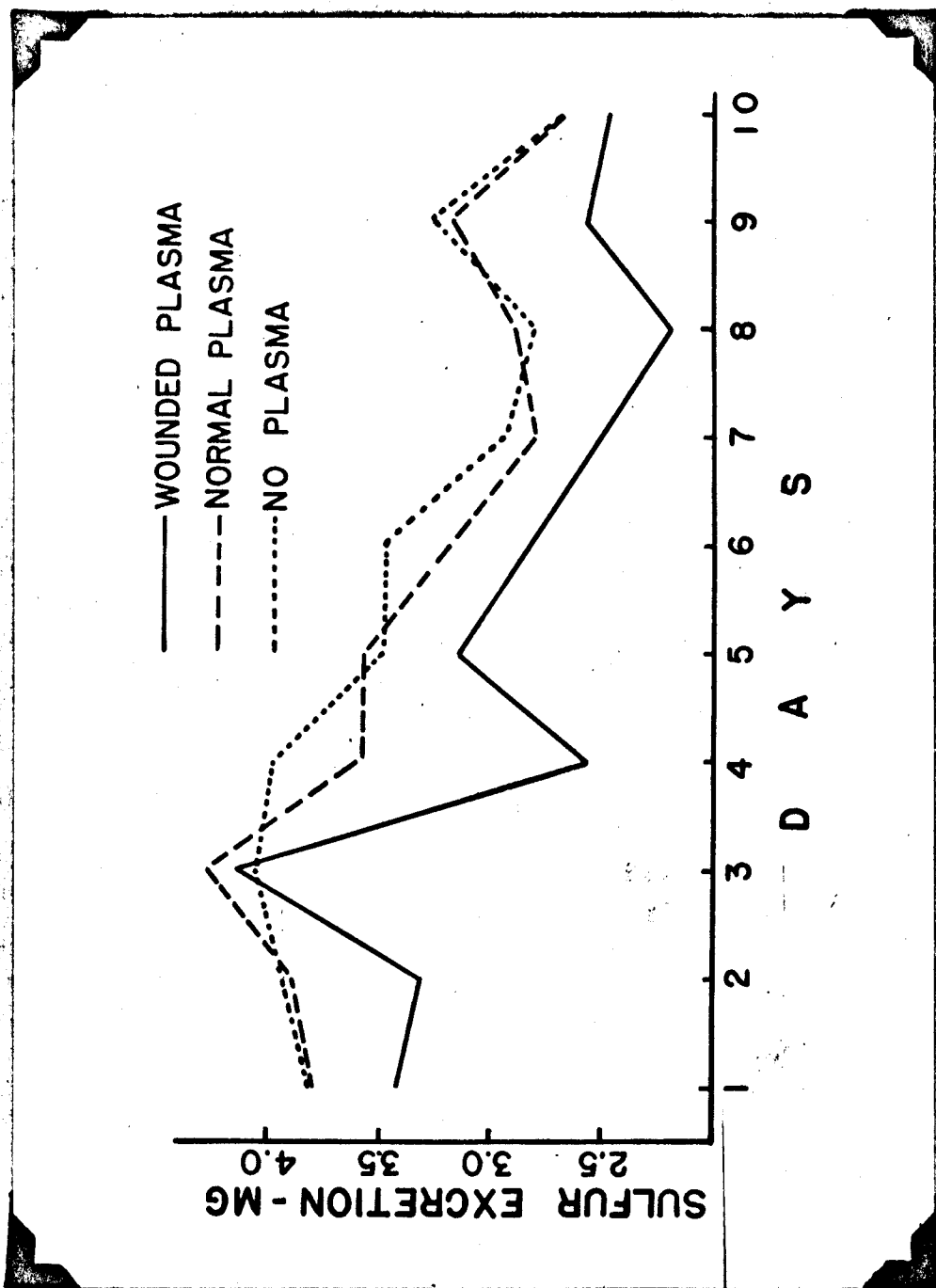
Plasma injections started on first day

Fig. IV

Excretion of total sulfur (mg./rat/day)

Plasma injections started on first day

The injection of the same amounts of plasma protein obtained from wounded animals could be expected to produce similar results. However, these animals excreted less nitrogen than did those which were injected plasma obtained from unwounded rats. It therefore appears that the presence of injured tissue in the donor animal has caused the release into the circulation of a factor or factors which have the ability, when injected into intact animals, to cause a reversal of the increased nitrogen metabolism produced by plasma without these factors.

Comparing the data on nitrogen and sulfur excretion it appears that in the animals treated with "normal" plasma, the increase in excretion of nitrogen is not followed by an proportional increase in the excretion of sulfur, since the amount of sulfur was approximately the same in the urines of Groups II and III. Although the excretion of nitrogen did not differ between Groups I and III, the excretion of sulfur seems to be lower in Group I than in the other two groups. It appears, therefore, that plasma from wounded animals contains some substance that stimulates the retention of sulfur.

To compare these findings to the sulfur and nitrogen metabolism in wounded animals, it has been shown that there is an increase in the excretion of nitrogen and a relative decrease in the excretion of sulfur after wounding. This has been interpreted to mean that there is an excess requirement for the sulfur amino acids during the healing process, the surplus nitrogen being excreted (Williamson and Fromm, 1955).

The results obtained from work with S^{35} labeled sulfur amino acids indicate that the rate of sulfur metabolism is increased during the healing of wounds. The uptake and loss of the S^{35} label in skin, muscle, and liver tissue

is greater in wounded rats than in uninjured animals. Wounded rats excrete larger amounts of S^{35} than do unwounded ones (Williamson and Fream, 1955).

In this experiment, data on excretion of total S^{35} and S^{35} specific activities in urine are shown in Table V and Fig. V. These data indicate that there is a very slight increase in the excretion of labeled sulfur in Groups I and II as compared to Group III. Apparently the administration of plasma proteins causes an increase in the metabolism of sulfur. Plasma from wounded rats, at least at the dose used, caused no further change.

At the termination of the experiment, the livers, kidneys, adrenals and ovaries of each animal were weighed. These weights are recorded in Table VI. The livers and kidneys in Group II were significantly heavier than those in the other two groups. This fact further seems to indicate that the administration of plasma has caused changes in the protein metabolism in these animals, and that plasma from wounded animals tends to reverse these changes. The administration of plasma proteins to protein deficient animals probably delays the depletion of tissue proteins, while some factors in blood of wounded animals tend to stimulate the breakdown of proteins in these organs in order that the breakdown products may be available for the healing process.

The results on excretion of sulfur and nitrogen by the animals in this experiment seem to add to the evidence that the hormonal composition is changed in plasma of wounded animals. However in this experiment, using unwounded animals, there was a retention of both nitrogen and sulfur, while wounding causes increased nitrogen excretion. Possibly the fact that the need for tissue repair was absent, altered the ratio of some amino acids required for protein synthesis. In addition the dosage of plasma used might play a role

TABLE V   38

The Excretion of S^{35} Labled Total Sulfur

and Specific Activity in Urine

Day	S^{35} Labled Total Sulfur*			S^{35} Specific Activity **		
	Group I	Group II	Group III	Group I	Group II	Group III
2	9,610	10,710	9,710	29.1	27.4	32.4
3	6,240	6,380	6,590	16.4	14.8	16.1
4	4,490	4,880	3,720	18.0	13.5	9.1
5	2,970	2,820	2,490	9.3	8.3	7.1
7	1,740	--	1,680	6.9	--	5.6
8	1,920	2,350	2,920	8.3	8.1	6.8
9	1,330	1,650	1,570	5.1	5.1	4.7
10	1,630	1,440	1,440	6.5	5.3	5.3

* Calculated in counts/minute/rat/day

** Specific activity = $\dfrac{\text{counts/minute/rat/day}}{\text{mg. S/rat/day}} \times 10^{-2}$
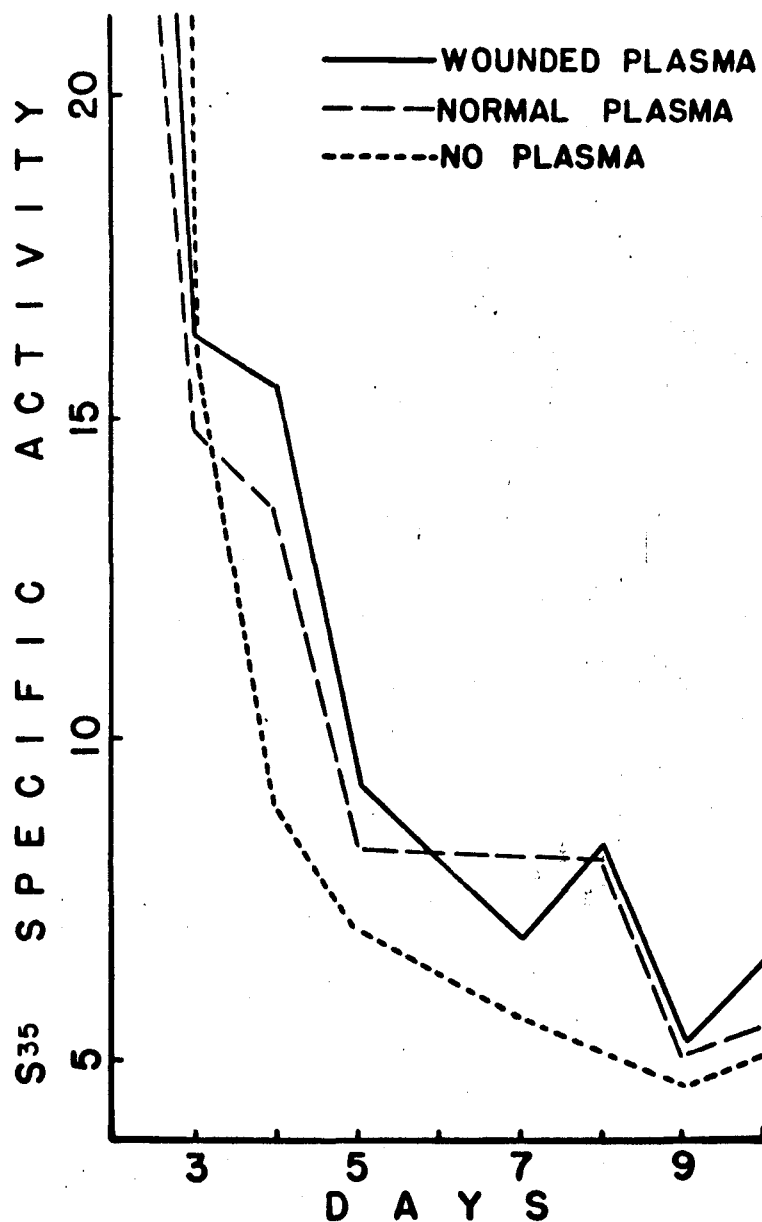
Fig. V

S^{35} specific activity= $\dfrac{\text{counts/minute/rat/24 hours} \times 10^{-2}}{\text{mg. S/rat/24 hours}}$

TABLE VI

Weight of Organs/100 gm. of Body Weight

	Group	No. of Animals	Weight	St. Error	
Kidneys	I	8	0.828 gm.	0.005	
	II	7	1.000 gm.	0.060	Group I–Group II; $p < 0.01$
	III	8	0.797 gm.	0.073	Group II–Group III; $p < 0.02$
Liver	I	8	3.20 gm.	0.17	
	II	7	4.85 gm.	1.05	Group I–Group II; $p < 0.01$
	III	8	3.57 gm.	0.27	Group II–Group III; $p < 0.02$
Adrenals	I	6	51.0 mg.	1.4	
	II	5	53.2 mg.	1.6	Group I–Group II; $p > 0.05$
	III	6	49.1 mg.	1.6	Group II–Group III; $p > 0.05$
Ovaries	I	8	57.8 mg.		
	II	5	57.6 mg.		
	III	8	58.6 mg.		
Body Weight	I	8	108 gm.		
	II	7	106 gm.		
	III	8	102 gm.		

in the results. Further, the work of Menkin (1943) indicates that injured cells are foci for gluconeogenesis.

The question still remains as to what the apparent changes in the hormonal composition of blood during the healing process are. The deviations from normal metabolism in injured animals, if at all, can only partly be ascribed to changes in adrenocorticoid production as initiated by the stress of trauma. Adrenalectomized animals maintained on constant doses of cortisone show the characteristic metabolic response to wounding (Ingle, 1954). Moore (1957) has suggested that wounding in some way may alter the intermediary metabolism of pituitary hormones and adrenal steroids. There seems to be a slight rise of the steroid hormones in the circulation of adrenalectomized animals maintained on constant dose of steroids after tissue damage, because of reduced clearance from the blood. This effect apparently is absent in stress of other types than tissue damage. These hormonal and metabolic changes might, therefore, be produced by some substance or substances released from the injured tissue.

With respect to the thyroid hormone, there is contradictory evidence. Perry and Gemmell (1949) found no change in the protein-bound iodine in patients after mild surgical trauma although inorganic iodine was increased in both urine and plasma, possibly suggesting increased destruction of thyroid hormone in the periphery. The work of Shipley and MacIntyre (1954) and of Engstrom and Markardt (1955) indicates that thyroid activity is not much altered by surgery. The work of Goldberg et al. (1956), on the other hand, indicates that there is a change in thyroid activity after wounding. These workers hypothesize that there is an interrelationship between thyroid and adreno-

cortical activity after trauma.

Trauma appears to be accompanied by a reduction in gonadotropic activity. This might be related to catabolic and nutritional changes associated with injury (Moore, 1957).

Growth Hormone

Hypophysectomy in all species observed is known to cause retardation of growth. Lack of the anterior pituitary has also been reported to cause retarded healing of wounds (Taubenhaus, 1950). The administration of purified growth hormone to either hypophysectomized or intact animals of many species results in an increased rate of growth. This may be extended also to the healing process, since the work of Taubenhaus (1950) indicates that the administration of growth hormone to hypophsectomized rats causes healing to approach towards normal, while administration of this hormone to intact animals produced an increased rate of formation of granulation tissue. Growth hormone, in many species of animals, causes changes in metabolism. The most apparent affect is an increased retention of nitrogen (as protein) in the tissues. This is accompanied by an increased metabolism of fat (Young, 1945; Greenbaum, 1953; Greenbaum, et al., 1953). It is unknown whether growth hormone affects wound healing through its action on protein metabolism in general, or if it also has a special affect on the healing process.

To compare the effect of growth hormone on nitrogen and sulfur metabolism in wounded and in unwounded animals, the following experiment was carried out. Four groups of six rats were allowed to become acclimated to a % casein diet for two days. Then each animal in two of the groups was inject-

ed with 0.5 ml. of a solution containing 0.25 mg. of purified growth hormone, dissolved in 0.05 M phosphate buffer at a pH of 8. The animals in the other two groups were each injected with 0.5 ml. of the buffer. These injections were made twice daily, and were continued throughout the course of the experiment. On the fourth day of the acclimatization period, one of the groups of rats which received growth hormone and one of those which did not, were anesthetized and wounded. The remaining two groups of animals were anesthetized, but were not wounded. Each animal was injected with 40 micro-curies of S^{35} labeled L-methionine. Twenty-four hour urine samples were collected throughout the course of the experiment.

The groups now were:

Group I   - unwounded

Group II   - unwounded and growth hormone

Group III - wounded

Group IV   - wounded and growth hormone

From the radically increased excretion of nitrogen, sulfur, and S^{35}, and loss in body weight by the animals in Group IV, it was suspected that some of the rats in this group must have been sick. Therefore, part of the experiment was repeated. This time three groups of animals were used. One group (Group IV-2) received 0.5 mg. growth hormone per day, the other group (Group IV-3) was given 1.0 mg. of growth hormone per day, while the animals in the third group (Group III-2)were injected with 0.5 ml. of phosphate buffer daily. All of these animals were wounded, and each was administered 25 micro-curies of S^{35} labeled L-methionine.

Data on the excretion of nitrogen by the animals in these two parts

of the experiment (Experiment A and Experiment B) can be seen in Fig. VI. It
appears that in unwounded rats growth hormone causes a relative retention of
nitrogen, while wounding causes increased nitrogen excretion. Growth hormone
administered to wounded animals causes a marked decrease in nitrogen excretion.
There does not seem to be any appreciable difference between the amounts of
nitrogen excreted by the group of wounded animals which were given 0.5 mg. of
growth hormone per day and those which received twice this amount of the
hormone.

The administration of growth hormone to wounded rats causes a degree
of nitrogen retention which appears to be greater than the retention caused by
this hormone in unwounded rats. From a comparison of the results of the two
parts of the experiment (Experiment A and Experiment B), it would appear that
the excretion of nitrogen is depressed by growth hormone to approximately the
same level in wounded and in unwounded rats. It is true that the growth hor-
mone treated unwounded animals in Experiment A excreted somewhat less nitrogen
than the growth hormone treated wounded rats in Experiment B. However, it
appears that the level of nitrogen excretion was also slightly lower in Group
III than in Group III-2, although these two groups of animals were identically
treated.

These data on nitrogen excretion, therefore, seem to indicate that
growth hormone, under the conditions of this experiment, depresses the excre-
tion of nitrogen to a definite level, regardless of whether the animals are
wounded or not. As has been mentioned before, wounding causes an increased
breakdown of tissue proteins and increased excretion of nitrogen, this probably
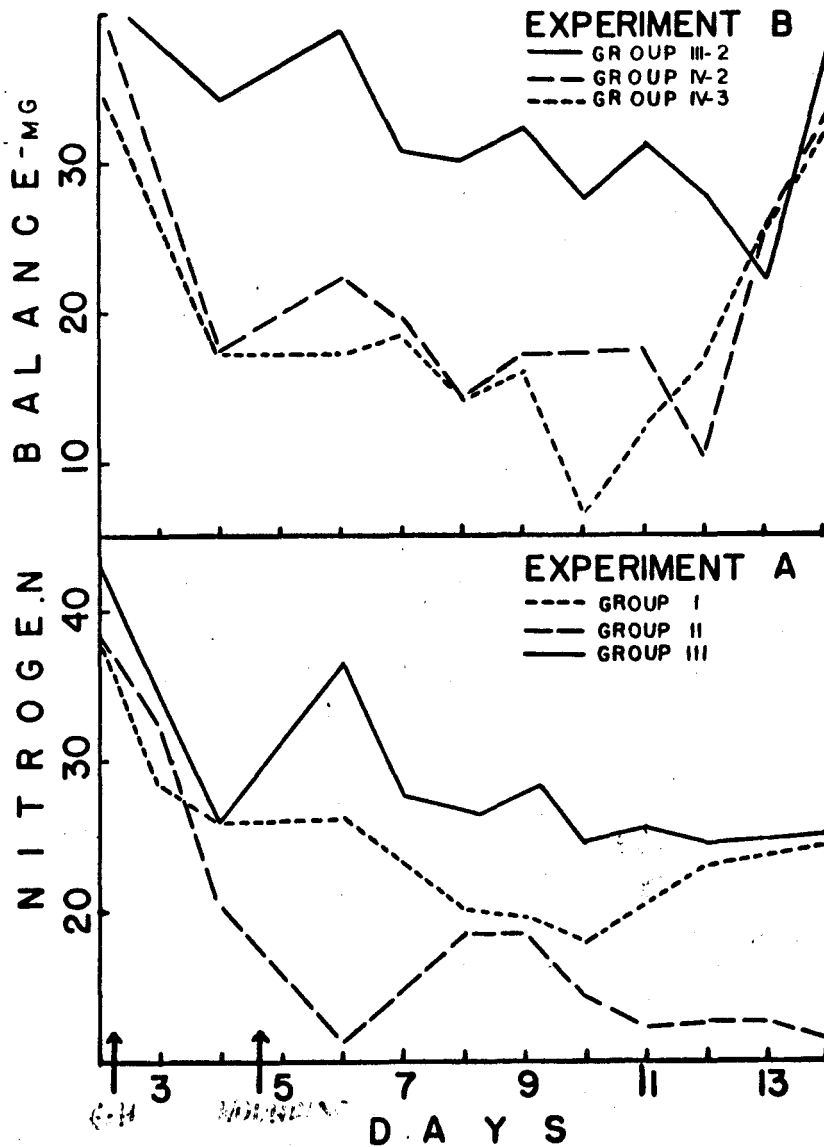being largely due to the increased need for sulfur amino acids. It appears

Fig. VI

Excretion of nitrogen (mg./rat/day)
Calculated as nitrogen balance by substracting the amount of nitrogen
contained in the diet from the total amount of nitrogen excreted

Experiment A
Group I   — unwounded
Group II   — unwounded & growth hormone
Group III — wounded

Experiment B
Group III-2 — wounded
Group IV-2 — wounded & GH (0.5 mg/day)
Group IV-3 — wounded & GH (1.0 mg/day)

that the administration of growth hormone has either the effect of inhibiting
this breakdown of tissue protein, or of causing a retention of the breakdown
products in the body. The work of Russell (1954) indicates that the nitrogen
retention caused by growth hormone is not the result of an inhibition of amino
acid catabolism. As a result of the influence of this hormone the rate of
removal of exogenous amino acids from the blood in increased and not decreased;
further, growth hormone causes an increased removal of these amino acids from
the circulation even in the absence of liver function. It also appears that
growth hormone does not affect the liberation of amino acids from body protein
(Roberman, 1950), since the hormone has been shown to cause a proportional
decrease in excretion of total nitrogen and isotopic nitrogen after administra-
tion of isotopic glycine. From this evidence it appears that the nitrogen
retention caused by growth hormone is not the result of an inhibition of amino
acid catabolism or protein breakdown, but rather a result of increased uptake
by the tissues of amino acids as proteins. It might, therefore, be supposed
that the products of the increased tissue breakdown, caused by the presence of
injured tissue in the body, are used for reincorporation into body proteins
under the influence of growth hormone.

Data on the excretion of total sulfur are presented in Fig. VII.
Only slightly lower excretion of sulfur can be noted in the unwounded growth
hormone treated animals than in the untreated ones. There is not much
difference between the amount of sulfur excreted by the wounded and unwounded
animals which were not treated with growth hormone. The growth hormone treat-
ed, wounded rats (Groups IV-2 and IV-3), seem to excrete less sulfur than the
wounded, growth hormone untreated animals for a few days after wounding and
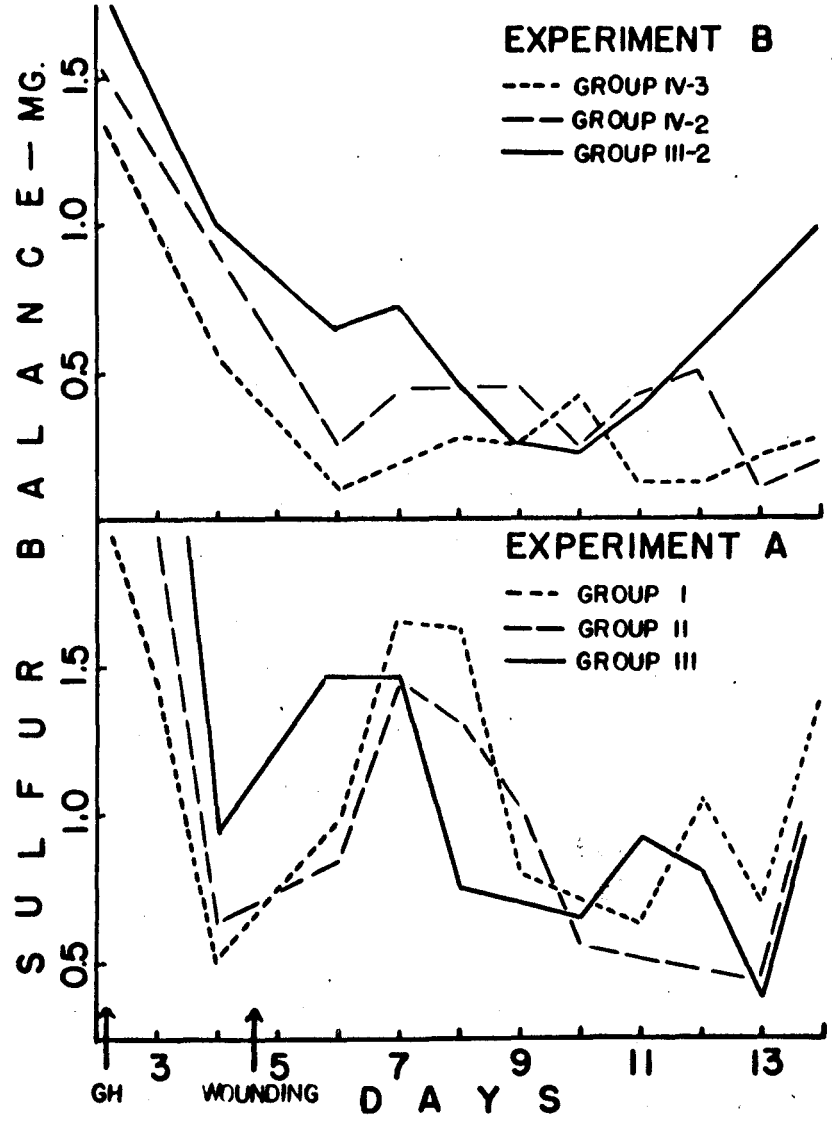
Fig. VII

Excretion of total sulfur (mg./rat/day)
Calculated as sulfur balance

Experiment A	Experiment B
Group I — unwounded	Group III-2 — wounded
Group II — unwounded & GH	Group IV-2 — wounded & GH (0.5mg./day)
Group III — wounded	Group IV-3 — wounded & GH (1.0mg./day)

then again during the latter part of the experiment. Figs. VIII and IX show data on the excretion of total S^{35} in the urine and the S^{35} specific activities. From these data it appears that, although the differences are slight, the normal, growth hormone treated rats excreted less S^{35} than did the normal animals, while the wounded rats excreted slightly more of the isotope. There was less S^{35} excreted by the growth hormone treated, wounded animals than the untreated, wounded rats during a few days after wounding and then again during the latter part of the experiment.

From these data it appears that the administration of growth hormone causes a slight decrease in the metabolism of the sulfur amino acids, or a retention of these amino acids in the body.

Comparing the data on the excretion of nitrogen and total sulfur, it can be seen that while the differences in sulfur excretion between the two groups in Experiment A are questionable, there are definite differences in the amounts of nitrogen excreted. In Experiment B there are definitely lower amounts of sulfur excreted by the growth hormone treated animals after the sixth or seventh day following wounding, while around this time the nitrogen excretion starts to increase and to approach that of the wounded, growth hormone untreated rats. The S^{35} activity also decreases at this time in the urine of the wounded growth hormone treated rats; the S^{35} specific activity does not change.

From these data the interpretation might be drawn that during the latter part of the experiment sulfur amino acids were used to a greater extent than before, and retained in the body in such a ratio to other amino acids that excess nitrogen had to be excreted. Further, it might be supposed that this
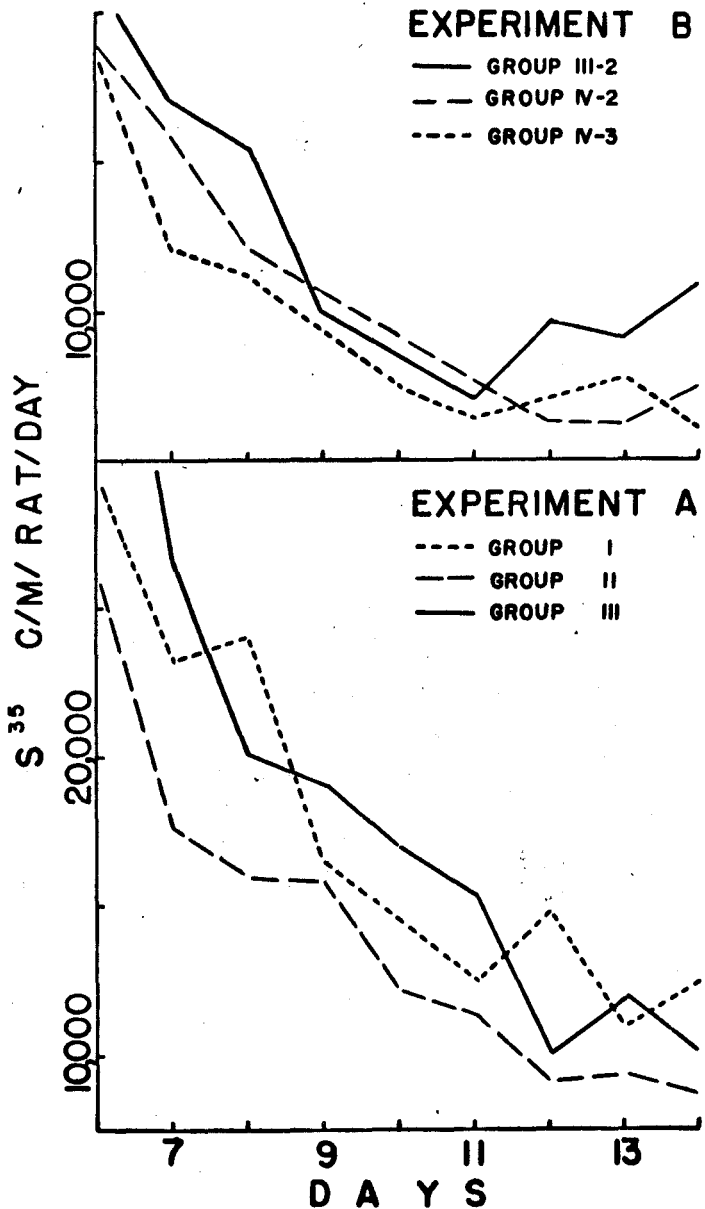
Fig. VIII

Total S^{35} in the urine (Counts/minute/rat/day)

Group I — unwounded
Group II — unwounded & GH
Group III — wounded

Group III-2 — wounded
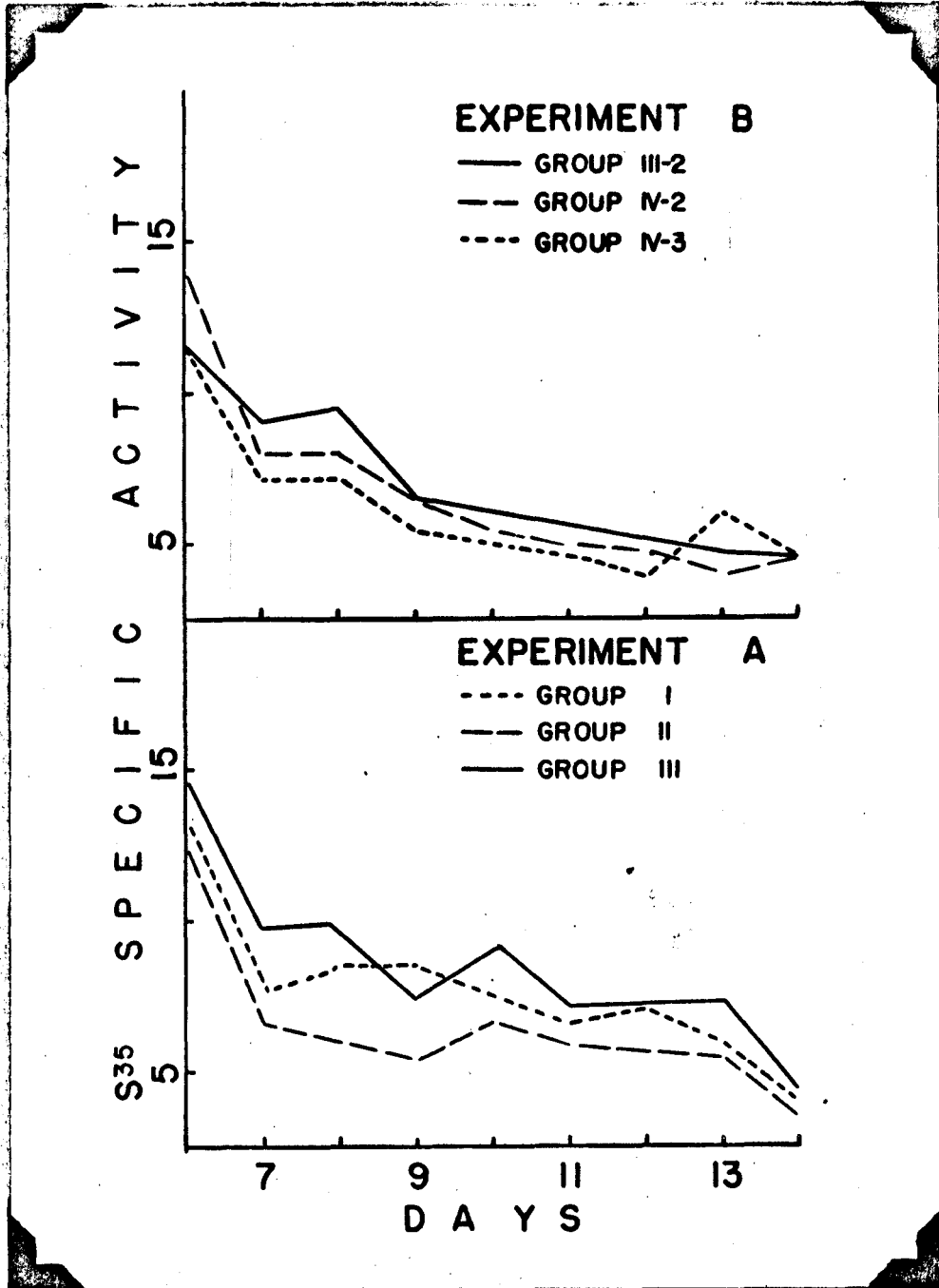Group IV-2 — wounded & GH (0.5mg./day)
Group IV-3 — wounded & GH (1.0mg./day)

Fig. IX

S^{35} specific activity in the urine

$$\frac{\text{counts/minute/rat/day} \times 10^{-3}}{\text{mg. S/rat/day}}$$

Group I — unwounded
Group II — unwounded & GH
Group III — wounded

Group III-2 — wounded
Group IV-2 — wounded & GH (0.5mg./day)
Group IV-3 — wounded & GH (1.0 mg./day)

retention of sulfur is associated with the healing process for no such changes can be seen in Experiment A. Timbenhaus (1950) has reported that while small doses (100) of growth hormone seem to favor healing in rats, large doses (500) inhibit it. The data of this experiment might indicate that the relatively large doses of growth hormone given the animals have inhibited healing during a period after healing, during which time the net retention of sulfur was low. This delay in healing might have been due to deficiency in amino acids required in the healing process caused by an increased synthesis of proteins in other than wound tissues. If the retention of sulfur, accompanied by an increase in nitrogen excretion, is enough basis for the supposition that processes of tissue regeneration are being carried on, then it could be assumed that the inhibition of healing by large doses of growth hormone has been overcome by the sixth or seventh day after wounding.

At the termination of the experiment wound and skin tissues were analyzed for nitrogen, sulfur, and S^{35}. The ratios of sulfur to nitrogen and the S^{35} specific activities in skin and wound tissue are shown in Table X. Perhaps no great weight may be attached to these results, because single, pooled samples were used for the analyses.

From the sulfur to nitrogen ratio in wound tissue, it appears that growth hormone has caused a decrease in the relative amount of sulfur in this tissue. This might give support to the supposition that healing was inhibited by growth hormone in this experiment, since the degree of healing is known to be related to the amount of sulfur amino acids in the wound tissue.

The S^{35} specific activity is higher in both skin and wound tissue in growth hormone treated animals. This may indicate that growth hormone has

TABLE X

The Sulfur to Nitrogen Ratio

And S^{35} Specific Activity in Skin and Wound Tissue

S/N

Experiment A Group	I	II	III	Experiment B Group III-2	IV-2	IV-3
Skin	0.279	0.149	0.261	0.350	0.270	0.342
Wound	--	--	0.470	0.930	0.800	0.730

S^{35} Specific Activity

Experiment A Group	I	II	III	Experiment B Group III-2	IV-2	IV-3
Skin	14.8	15.8	9.7	17.3	24.1	21.3
Wound	--	--	21.3	47.0	51.5	53.0

caused either an increased uptake or a decreased turnover of the sulfur amino acids in these tissues. From these data no difference in response to growth hormone between skin and wound tissue can be seen.

SUMMARY

The purpose of this thesis is to investigate whether some of the metabolic changes which have been reported by Williamson et al. (1952-1955) to accompany wound healing, could be brought about or influenced by hormonal factors.

It was desired to investigate whether changes in the metabolism of sulfur and nitrogen could be brought about by hormonal factors released in the circulation by the wounding stimulus. To study this, plasma from wounded animals was injected into unwounded, protein deficient rats. It was found that control animals which were injected with plasma from unwounded rats, excreted more nitrogen than did animals which were given plasma from wounded rats or those which did not receive any plasma. This might indicate a stimulation of nitrogen retention in the animals receiving plasma from wounded donors. The retention of sulfur also appeared to be stimulated. As evidenced by the excretion of S^{35}, the administration of plasma proteins probably caused a slight increase in the metabolism of sulfur. The changed hormonal composition of plasma from wounded rats caused no further change.

It was concluded that the changes in nitrogen and sulfur metabolism caused by the injection of plasma from wounded animals add to the evidence that the hormonal composition is changed in the blood of wounded animals. This plasma apparently contained some component or components which are lacking in

plasma of unwounded animals, and which have the ability to increase anabolic reactions in the body.

The affect of the pituitary growth hormone on the metabolism during wound healing was also studied. Growth hormone at the relatively large doses used in this experiment appeared to depress the excretion of nitrogen to a definite level, regardless of whether the experiment animals were wounded or not. The hormone caused a decrease in the excretion of total sulfur in wounded animals after the sixth day after wounding.

BIBLIOGRAPHY

Arey, L. B. (1936) Wound Healing, Physiol. Rev. 16, 327.

Asboe-Hansen, O. (1950) The Variability in the Hyaluronic Acid Content of the Dermal Connective Tissue Under the Influence of Thyroid Hormone. Acta Dermato-venereol. 30, 221.

Asboe-Hansen, O., Iversen, K., and Wichman, R. (1952) Malignant Exopthalmos; Muscular Changes and Thyrotrophin Content in Serum, Acta Endocrinol. 11, 376.

Asboe-Hansen, O. (1954) Autoradiographic Evidence of Cortisone Action on Mast Cells in Experimental Skin Tumors, Cancer Res. 14, 94.

Asboe-Hansen, O. (1954) Connective Tissue in Health and Disease, Munksgaard, Copenhagen.

Auerbach, E., and Doljanski, L. (1954) Effect of Cell Growth Activitating Tissue Extracts, Parenterally Applied, on Experimental Wounds Proc. Soc. Exp. Biol. Med. 58, 111.

Auerbach, E. (1952) An Inhibiting Effect Observed in the Course of Normal Wound Healing, Proc. Soc. Exp. Biol. Med. 81, 294.

Beach, E. F., Teague, M. D., Hoffman, O. D., Munks, B., Hummel, F. C., Williams, H. H., Macy, I. G. The Sulfur Metabolism of Children, J. Nutrition, 24, 257.

Boström, H. and Gardell, S. (1953) Uptake of Sulphates in Mucopolysaccharides Esterified With Sulphuric Acid in the Skin of Adult Rats After Intraperitoneal Injection of S^{35} Labelled Sodium Sulphate Acta Chem. Scand. 7, 316.

Bowes, J. H., Elliot, R. G. and Moss, O. A. (1953) Some Differences in the Composition of Collagen and Extracted Collagens and their Relation to Fibre Formation and Dispersion, in Nature and Structure of Collagen Butterworth, London p. 199.

Castor, W. C. and Baker, B. L. (1950) Local Action of Adrenocortical Steroids on Epidermis and Connective Tissue of the Skin, Endocrinology, 47, 234

Catchpole, H. R. (1957) Role of the Ground Substance in Wound Healing, in M. B. Williamson (Ed.) The Healing of Wounds, McGraw-Hill, Inc.

Cook, E. S., and Fardon, J. (1942) The Wound Hormone Concept in Wound Healing, Surg. Gynec. Obstet., 75, 220.

Danielli, J. F., Fell, H. B. and Kodicek, E. (1945) The Enzymes of Healing Wounds. II: The Effect of Different Degrees of Vitamins C-Deficiency on the Phosphatase Activity in Experimental Wounds in the Guinea Pig, Brit. J. Exper. Path., 26, 267.

Dunphy, J. E. and Udupa, K. N. (1955) Chemical and Histochemical Sequences in the Normal Healing of Wounds, N. E. J. Med., 253, 847.

Edwards, L. C. and Dunphy, E. J. (1957) "Methionine in Wound Healing During Protein Starvation" in M. B. Williamson (Ed.) The Healing of Wounds, McGraw-Hill, Inc.

Engstrom, W. W. and Markardt, B. (1955) The Effects of Serious Illness and Surgical Stress on the Circulating Thyroid Hormone, J. Clin. Endocrinol. and Metab. 15, 953.

Findlay, C. W. and Howes, E. L. (1952) The Combined Effect of Cortisone and Partial Protein Depletion on Wound Healing, New Engl. J. Med., 246, 597

Forbes, R. M. (1954) Studies on the Influence of Antibiotics and Methionine on Nitrogen Utilization and Basal Metabolism of the Growing Male Albino Rat, J. Nutrition, 53, 275.

Fromm, H. J. and Nordlie, R. C. (1956) Experimental Injury and Sulfur Amino Acid Metabolism, J. Biol. Chem. 223, 737.

Gerarde, H. W. and Jones, M. (1953) The Effect of Cortisone on Collagen Synthesis in vitro, J. Biol. Chem., 201, 553.

Gersh, I. and Catchpole, H. R. (1949) The Organisation of Ground Substance and Basement Membrane and Its Significance in Tissue Injury, Disease and Growth, Am. J. Anat., 85, 457.

Goldenberg, I. S., Lutwak, L., Rosenbaum, P. J. and Hayes, M. A. (1956) Thyroid Activity During Operation, Surg. Gynecol. Obstet., 102, 129.

Greenbaum, A. L. (1953) Changes in Body Composition and Respiratory Quotient of Adult Female Rats Treated with Growth Hormone, Biochem. J., 54, 400.

Groenbaum, A. L. (1953) The Influence of Pituitary Growth Hormone on the Catabolism of Fat Biochem. J. 54, 413.

Highberger, J. H., Gross, J., and Schmitt, F. O. (1951) The Interaction of Mucoprotein With Soluble Collagen; An Electron Microscope Study, Proc. Nat. Acad. Sci., 37, 286.

Holden, M. and Adams, L. B. (1957) Inhibitory Effects of Cortisone Acetate and Hydrocortisone on Growth of Fibroblasts, Proc. Soc. Exper. Biol. Med., 95, 364.

Howes, E. L. (1954) "The Connective Tissues in Wound Healing" in G. Asboe-Hansen (Ed.) Connective Tissue in Health and Disease, Copenhagen, Munksgaard.

Kulonen, E. (1954) "Recent Advances in the Chemistry of Collagen" in G. Asboe-Hansen (Ed.) Connective Tissue in Health and Disease, Copenhagen, Munksgaard.

Ingle, D. J. (1954) J. Clin. Endocrinol. and Metab., 14, 1272.

Layton, L. L. (1950) In Vitro Sulfate Fixation by Granulation Tissue and Injured Muscle Tissue from Healing Wounds, Proc. Soc. Exp. Biol. Med., 73, 570.

Layton, L. L. (1951) Effect of Cortisone Upon Chondroit in Sulfate Synthesis by Animal Tissues, Proc. Soc. Exper. Biol. Med., 76, 596.

Li, C. H. and Evans, H. M. (1948) The Biochemistry of Pituitary Growth Hormone, Recent Progress in Hormone Research, 3, 1.

Loewi, G. nad Meyer, K. (1958) The Acid Mucopolysaccharides of Embryonic Skin, Biophys. et Biochem. Acta., 27, 453.

Ludwig, A. W., Boas, N. F., and Soffer, L. J. (1950) Role of Mucopolysaccharides in Pathogenesis of Experimental Exophthalmos, Proc. Soc. Exper. Biol. Med., 73, 137.

Menkin, V. (1943) Gluconeogenesis and Cellular Injury. A Further Inquiry Into the Mechanism Involved in Diabetes Enhanced by Inflammation, Am. J. Physiol., 130, 396.

Menkin, V. (1956) Biochemical Mechanisms in Inflammation, Charles Thomas, Publ.

Meyer, K. (1947) The Biological Significance of Hyaluronic Acid and Hyaluronidase, Physiol. Rev., 27, 335.

Meyer, K., Davidson, E., Linker, A., and Hoffman, P. (1956) The Acid Mucopolysaccharides of Connective Tissue, Biochem. et Biophys. Acta., 21, 506.

Moltke, E. (1955) Wound Healing Influenced by Thyroxine and Thyrotrophic Hormone, Proc. Soc. Exper. Biol. Med., 88, 596.

Moltke, E. (1956) Role of Ascorbic Acid in Wound Healing Influenced by D, L- Thyroxine, Acta Endocrinol., 23, 105.

Moltke, E. (1957) Uptake of S^{35}-Sulphate by Healing Wounds, Acta Endocrinol., 25, 179.

Moore, F. D. (1957) Endocrine Changes After Anesthesia, Surgery and Unanesthotized Trauma in Man, in Recent Progress in Hormone Research, Vol. 13, p. 511.

Morgan, J. F., and Morton, J. (1955) Studies on the Sulfur Metabolism of Tissues Cultivated in vitro, J. Biol. Chem., 215, 539.

Morris, H. P., Dubnik, C. S. and Dunn, T. B. (1945) Technique for Studying Epitholization of Paired Skin Wounds in Rats, With Observations on the Influence of Dietary Protein, J. Nat. Cancer Inst., 5, 271.

Needham, A. E. (1952) Regeneration and Wound Healing, London, Methuen & Co., Ltd.

Neuberger, A. (1955) Symphosia Soc. Exptl. Biol., No. 9, Fibrous Proteins and Their Biological Significance, p. 72.

Orekhovitch, V. N. (1950) Ukrain. Biokhim. Zhur., 22, 456.

Perez-Tamayo, R., and Ihnen, M. (1953) The Effect of Methionine in Experimental Wound Healing, Am. J. Pathol., 29, 233.

Perry, W. F. and Gommell, J. P. (1949) Effect of Surgical Operations on the Excretion of Iodine, Corticosteroids, and Uric Acid, Can. J. Research, 27 E, 320.

Pirani, C. L., Stepto, R. C. and Sutherland, K. (1951) Desoxycorticosterone Acetate and Wound Healing, J. Exper. Med., 93, 217.

Ragan, C., Howes, E. L., Plotz, C. M., Meyer, K. and Blunt, J. W. (1949) Effect of Cortisone on Production of Granulation Tissue in the Rabbit, Proc. Soc. Exp. Biol. Med., 72, 718.

Rice, F. A. (1956) Chondroitin Sulfate and Hyaluronic Acid in Syphilomas of Cortisone-Treated Rabbits, Science, 124, 275.

Robertson, W., van B. and Hinds, H. (1956) Polysaccharide Formation in Repair Tissue During Ascorbic Acid Deficiency, J. Biol. Chem., 221, 791.

Sandblom, Ph. (1949) The Effect of Injury on Wound Healing, Ann. Surg., 129, 305.

Schiller, S. and Dorfman, A. (1957) The Metabolism of Mucopolysaccharides in Animals: the Effect of Cortisone and Hydrocortisone on Rat Skin, Endocrinology, 60, 376.

Sayers, G. (1957) Factors Influencing ACTH Level in the Blood, Hormones in Blood; Ciba Foundation Colloquia on Endocrinology, 11, 130.

Selyo, H. (1955) Dependence Upon NaCl and Mineralo-Corticoids of the Normal Responsiveness to Somatotrophic Hormone, Am. J. Physiol., 181, 367.

Shapiro, R., Taylor, B. and Taubenhaus, M. (1951) Local Effects of Cortisone on Granulation Tissue and the Role of Denervation and Ischemia, Proc. Soc. Exp. Biol. Med., 76, 854.

Shipley, R. A. and MacIntyre, F. M. (1954) Effect of Stress, Thyrotropic Hormone (ACTH) on the Level of Hormonal Iodine[131] of Serum, J. Clin. Endocrinol. and Metab., 14, 309.

Smelser, G. K. (1938) Treatment of Experimentally Produced Exopthalmos With Thyroxin and Other Iodine Compounds, Am. J. Opth., 21, 1208.

Spain, D. M., Molomut, N. and Haber, A. (1950) Biological Studies of Cortisone in Mice, Science, 112, 335.

Stearns, M. L. (1940) Studies on the Development of Connective Tissue in Transparent Chambers in the Rabbit's Ear. II, Am. J. Anat., 67, 55.

Taubenhaus, M. and Amromin, G. D. (1949) Influence of Steroid Hormones on Granulation Tissue, Endocrinology, 44, 359.

Taubenhaus, M. and Amromin, G. D. (1950) The Effects of the Hypophysis, Thyroid, Sex Steroids, and the Adrenal Cortex Upon Granulation Tissue, J. Lab. Clin. Med., 36, 7.

Taubenhaus, M., Taylor, B. and Morton, J. V. (1952) Hormonal Interaction in the Regulation of Granulation Tissue Formation, Endocrinology, 51, 183.

Taubenhaus, M. (1953) Influence of Cortisone Upon Granulation Tissue and Its Synergism to Other Hormones, Ann. N.Y. Acad. Sci., 56, 666.

Taubenhaus, M., Jacobson, M., Morton, J. V., and Levine R. (1953) Parallel Inhibition of Granulation Tissue by Cortisone, Hydrocortisone, Dibonamine, and Banthine, Proc. Soc. Exper. Biol. Med., 84, 646.

Teir, H. and Kiljunen, A. (1951) Existence of a Growthpromoting Factor in the Skin of the White Rat, Ann. Chir et Gynaec. Fenniae, 1, 61.

Teir, H. (1956) Some New Observations on the Mitosis-Stimulating and Tumor-Inducing Effects of Intraperitoneally Injected Tissue Suspensions in Rats, Acta Pthol. et Microbiol. Scand., 111, 87

Toennies, G. and Bakay, B. (1953) Photonopholometric Microdetermination of Sulfate and Organic Sulfur, Anal. Chem., 25, 160.

Udupa, K. N., Woessner, J. F. and Dunphy, J. E. (1956) The Effect of Methionine on the Production of Mucopolysaccharides and Collagen in Healing Wounds of Protein-Depleted Animals, Surg. Gynecol. Obstet., 102, 639.

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. Med., 77, 302.

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

Williamson, M. B. and Fromm, H. J. (1953) Utilization of Sulfur Amino Acids During Healing of Experimental Wounds, Proc. Soc. Exp. Biol. Med., 83, 329.

Williamson, M. B. and Fromm, H. J. (1954) Excretion of Sulfur During Healing of Experimental Wounds, Proc. Soc. Exp. Biol. Med., 87, 366.

Williamson, M. B. and Fromm, H. J. (1955) The Incorporation of Sulfur Amino Acids Into the Proteins of Regenerating Wound Tissue, J. Biol. Chem., 212, 705.

Zachariae, L. and Moltke, E. (1954) Influence of Hydrocortisone on Mast Cells of Normal Skin and Healing Wounds in the Rabbit, Acta endocrinol., 16, 300.

The thesis submitted by Lilita Straumanis has been read and approved by three members of the faculty of the Graduate School.

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

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

_____   _____
Date   Signature of Adviser