THE SUBCUTANEOUS INFLAMMATORY RESPONSE TO THREE SOLUBLE AMINO ACIDS IN PROTEIN DEPLETED RATS

By

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CHAPTER I

INTRODUCTION

An understanding of the factors involved in the healing of wounds is of fundamental importance to the surgeon. This is especially true when dealing with aged or debilitated patients. With such patients all available means must often be used to promote healing.

An interest in the healing of wounds has appeared in the field of cancer research. Some of the broader aspects of cancer research are concerned with the study of factors affecting the growth of normal tissue. A knowledge of the basic factors involved in the inflammatory reaction is also vital to research on many diseases other than cancer. A vast number of diseases are characterized by an inflammatory reaction of some form.

It is well established that protein metabolism plays an important role in the healing of wounds. However, the exact mechanism by which protein promotes healing is not well understood. Some investigators have demonstrated that certain amino acids, particularly methionine, have a beneficial influence on fibroplasia under conditions in which there is a deficiency of dietary protein. In most of the previous work the amino acids were administered parenterally. In this study the solutions were applied directly to the wounds.

Specifically, the purpose of this investigation was to determine

if the inflammatory reaction was altered by the local introduction of certain amino acids. The study includes both the acute response to an irritant and the healing process. The work was carried out in both normal and protein depleted animals. Plastic sponges placed in the subcutaneous tissue served as "wounds." The plastic sponges facilitated the introduction of the amino acids. The response was measured by histologic examination of the subcutaneous implants and adjacent tissue.

In this study there was particular interest in the response to methionine. The literature indicates that the sulfhydryl group plays an important role in wound healing. The other amino acids selected were lysine and arginine. Both of these are essential in the diet and are free of sulfhydryl activity. There is some variation in the results obtained by previous investigators in the use of lysine and arginine. All three of the amino acids chosen are among those that are the most soluble in water. This permits a relatively high concentration in the test solution.

Early in the investigation an attempt was made to measure both the fibroblast proliferation and the epithelial regeneration by means of sponge implants placed in the skin of guinea pigs. These efforts were unsuccessful because the proliferating cells did not invade the implant; rather, healing progressed under the implant with the implant acting more or less as a scab. This was partially attributed to the fact that the sponge became dry and hard upon exposure to air. In order to overcome drying of the sponge the wound was covered with a film of spray-on plastic bandage (Aeroplast[1]). The plastic bandage

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[1] Aeroplast is the trade name of a spray-on plastic bandage and protective surgical dressing manufactured by the Aeroplast Corporation, Dayton, Ohio.

did not adhere well to the moist surface of the sponge and healing continued to occur beneath the sponge.

Following this, implants were made within the muscles of rabbits, hamsters, and guinea pigs. The aim of intramuscular implants was to avoid contact with subcutaneous fat deposits that might delay healing. The implants were difficult to insert within the muscles of the smaller laboratory animals and only a small implant could be used. There was the additional objection of considerable compression of the implant resulting in distortion of the implant and a loss of solution with which the implant was saturated.

After intramuscular implants were found to be undesirable, subcutaneous implants were decided upon. It was thought that a more accurate measurement of the connective tissue proliferation might be obtained if the direction of infiltration could be limited to the two ends of the implant and the depth of penetration observed. This was attempted by inserting a cylindrical sponge implant 3 mm. in diameter and 1 cm. in length into a "sleeve" of Tygon[2] tubing of similar dimensions. The formulation chosen was that specifically manufactured for surgical and pharmaceutical use. This formulation is colorless, flexible, chemically inert, and non-toxic. It is used in heart lung machines, artificial kidneys, tumor perfusion machines, and similar apparatus. In spite of the inertness of the Tygon tubing, invasion of the sponge by fibroblasts did not occur. Instead a thin capsule of fibrous connective tissue formed around the outside of the tubing.

In selecting material for the implants a number of features were

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[2]Tygon is a plastic material manufactured by the U. S. Stoneware Company, Akron, Ohio.

considered. It was desirable that the material be chemically inert, non-irritating to tissues, tolerate autoclaving, be pliable when moist yet maintain its shape to some degree when in tissues, and have uniform pore size. In the early preliminary work, plastic household sponge, thought to be polyurethane, was used. This material possessed most of the desirable characteristics outlined, especially uniform pore size, however it was objectionable in that the sponge was dissolved during the process of staining the slides. Furthermore, the chemical composition was not definitely known. In later work, cigarette filters were used. These were unsatisfactory because of the tendency to break apart when wet. Again the exact chemical composition was not definitely known. Eventually, Ivalon surgical sponge was tried and found to be reasonably satisfactory in all respects. This material has been widely used experimentally for prosthesis and has been used by other investigators for subcutaneous implants.

Implants of various shapes were tried including square, rectangular, and cylindrical. A cylindrical implant 5 mm. in thickness and 9 mm. in diameter was found to be preferred. An implant of this design could be placed in the tissue with a flat surface down and all points on the perimeter would be an equal distance from the center.

Loss of test solution from the implants was observed during the act of implanting. In an effort to overcome this, the implants were placed in an oven after being saturated with amino acid solution and held at 60° C. until completely dry. While drying, the amino acid crystals migrated toward the periphery of the implant and formed a layer on the surface of the sponge. The central portion of the implant was found to be completely devoid of amino acid. A wet sponge

was therefore considered more desirable than a dry one because of more uniform distribution of the amino acid solution.

New Zealand White rabbits were used for the initial subcutaneous implants. The relatively large size of the animal permitted several implants to be made within a single individual. Since wounds heal at a different rate in various parts of the body, only one implant could be placed on each side when accurate interpretations were to be attempted. Because of this and other problems encountered in the surgical procedures, white rats were selected as the experimental animal. With rats, there was the additional advantage that a commercially prepared protein depleting diet was available.

CHAPTER II

REVIEW OF SELECTED LITERATURE

Methods of Measuring the Inflammatory Response

A number of techniques have been devised to observe and measure the inflammatory response of tissue. There are certain limitations and advantages to each of the methods and the technique of choice would seem to depend on the particular problem under consideration.

One method consists of the formation of a standard skin wound (Dann, Glucksmann, and Tansley, 1941). The progressive closure of this wound can be measured and the composition of the regenerating tissue can be studied chemically and histologically. A modification of this technique is reported by Abercrombie, Flint, and James (1954) and by Grillo, Watts, and Gross (1958). With this method a square of certain dimensions is measured on the animal's skin. The four points of the square are tattooed with india ink, and additional points are tattooed midway between the original points making a total of eight tattoo marks. A wound is made just inside the boundaries of the tattooed square removing the skin and panniculus carnosus. The tattoo points mark the advancing edges of the wound. With an open skin wound of this type contraction of the margins accounts for a large proportion of the closure of the wound and this does not measure proliferation of fibroblasts and epithelium.

In order to eliminate the effect of contraction, Morris, Dubnik,

and Dunn (1945) devised a perforated aluminum disc to be attached to the rump of experimental animals. The disc is sutured to the skin and sealed with collodion. A circular portion of skin is removed through an aperture in the center of the disc. Sterilized dressings are used throughout the healing period to protect the wound.

Development of tensile strength may be used as a measurement of the rate of healing of experimental wounds. In the experiments of Localio, Morgan, and Hinton (1948) the tensile strength of ventral midline incisions was measured by distending the peritoneal cavity with air and determining the bursting pressure in millimeters of mercury. A similar technique was used by Harvey and Howes (1930, 1933) with incisions in the stomach of rats.

Kieler (1953) used tissue culture technique to study the influence of amino acids on mitotic activity of fibroblast cultures. Fibroblast cultures were grown in a dialyzed medium and various amino acids added to the medium. Although the growth supporting effect of the medium is not completely eliminated by dialysis, it is reduced to such an extent that a mitotic promoting effect of additives can be demonstrated.

Another method for observing the inflammatory response is by the use of implanted inert material. The material is usually implanted subcutaneously and the degree of infiltration of inflammatory cells determined on histologic sections of the material. Among the materials used are polyvinyl sponge, Bing (1955), Edwards, Sarmenta, Hass (1960), Edwards, Pernokas, Dunphy (1957), Grindlay and Waugh (1951), tantalum wire mesh, Schilling, Favata, Radakovich (1953), and surgical cellulose sponge, Corcoran (1958). Essentially the same healing processes occur with a closed wound of this type as occurs with an open wound except

there is an absence of contraction, scar formation, and epithelial regeneration.

Factors Influencing the Rate of Healing

There are a number of factors that influence the rate of wound healing. Important in this respect are species of animal, age of the individual, diet of the animal, size of the wound, and location of the wound.

It is recognized that there is a difference in the rate of healing in various species of animal. In studies of wound healing in the guinea pig, rat, and pigeon, Loeb (1920) found regeneration rate greatest in the guinea pig and least in the pigeon. The rat lies between the guinea pig and pigeon in regeneration of tissue.

The rate of healing in young animals is greater than in mature animals. Howes and Harvey (1932) found healing of stomach wounds to be more rapid in young rats than in adults because fibroplasia began earlier and was less retarded. The velocity curve of fibroplasia in the healing of wounds in young rats reached its end point three days ahead of a similar curve for adults.

Size of the wound has been found to influence the rate of healing. In observing the healing of wounds in the skin of rabbits, Young, Fisher, and Young (1941) confirmed that large wounds close at a greater rate than smaller wounds. In general the rate of closure was directly proportional to the area of the wound. Their work also indicated that secondary wounds close at a greater rate than primary wounds. This was determined by comparing closure rates of primary wounds with those of wounds made ten to fourteen days after the initial incision.

The location of the wound has an effect on the healing rate of wounds. Young, Cruickshank, and Martin (1946) observed that rabbit wounds in the anterior half of the back heal more rapidly than those in the posterior half. As might be expected, wounds on the right and left side heal at an equal rate (Morris, Dubnik, Dunn, 1945).

The level of nutrition plays an important role in the healing process. The vitamins and protein are of prime importance in this respect. Dietary protein has two principal functions in the healing of wounds. First, the plasma proteins maintain the colloidal osmotic pressure of the blood and secondly, it provides the essential amino acids for growth.

There is some uncertainty as to the exact mechanism of delayed wound healing on a low protein diet. Rhoades, Fliegelman, and Panzer (1942) found that fibroplasia occurs in the hypoproteinemic animal if the colloidal osmotic pressure of the plasma is maintained with intravenous acacia. This occurs even if chronic hypoproteinemia is produced so as to deplete the stores of labile proteins. Two explanations of the effect of acacia have been presented. One is that the relation of hypoproteinemia to wound healing is mainly nutritional. The administration of acacia makes it possible for more protein to be withdrawn from the plasma for tissue building purposes. The other explanation of the effect of acacia is that the relation of hypoproteinemia to wound healing is mainly on an osmotic basis. When cell cultures are surrounded by an overabundance of fluid of low viscosity, cell motility is retarded (Thompson, Ravdin, Frank, 1938).

Complete starvation for a short period of time does not necessarily retard wound healing in the adult animal. Howes, Briggs, Shea, and

Harvey (1933) found that in adult rats the rate of healing in wounds of the stomach was not appreciably affected by complete starvation. On the other hand, the healing of wounds in young rats was decidedly retarded by giving only one-half the required amount of an adequate diet. One possible explanation for the healing rate of adult rats on a starvation diet is that in the final days of starvation the metabolism is almost if not entirely protein, therefore there would be an increase in the available amounts of amino acids.

Morris, Dubnik, and Dunn (1945) observed the rate of healing with rats on a low and high protein diet. Rats on a low protein diet were fed this diet for two weeks prior to experimentation. Two weeks was considered to be the minimum amount of time for the depletion of normal protein reserves in the body of experimental animals. A significantly slower rate of healing was obtained in animals fed a low protein diet. This was partially due to a shorter lag period in the group on the high protein diet. Histologically, the wounds of rats receiving the low protein diet were distinguished from those on a high protein diet by excessive edema, poor organization, the deposition of a granular intercellular substance, and tardy development of fibroblasts and mature collagen fibers. The addition at the time the wound was made of any one of the three essential amino acids, valine, tryptophane, and lysine to the low protein diet had no beneficial effect on retarded epithelization.

Thompson, Ravdin, and Frank (1938) demonstrated a high incidence of wound dehiscence in dogs with low serum protein levels. Tissues in the hypoproteinemic dogs exhibited marked edema and definite delay in fibroblastic proliferation. Even after fourteen days fibroblasts had not appeared in numbers expected in a wound of a normal animal. Localio,

Chassim, and Hinton (1958) found tissue protein depletion to be an important factor in wound dehiscence in human patients.

It appears that the velocity of growth in a healing wound may be even greater on a high protein diet than on a standard diet. Harvey, and Howes (1930) reported that a high protein diet (68.7% protein) enhanced the rate of increase of tensile strength throughout the period from the fifth to the ninth day and that maximum strength was reached two days earlier than in the case of a standard diet (13.8% protein).

Some investigators have found that when low protein diets are supplemented with methionine an increase in fibroplasia occurs. Localio, Morgan, and Hinton (1948) divided rats into three groups. One group served as normal controls and two groups were placed on protein depleting diets for five weeks before surgery. One of the protein depleted groups received daily injections of 150 mg. of methionine subcutaneously for ten days following surgery. From this it was concluded that protein depletion in rats causes a prolonged lag period, a slowed proliferative period, and a delay of final healing and that parenteral administration of methionine to protein depleted rats shifts the curve of wound healing toward normal.

The effect on experimental wound healing of a short term protein deficiency supplemented by methionine was studied in the rat by Tamayo and Ihnen (1953). Animals given a normal diet, a protein deficient diet, and a protein deficient diet supplemented by valine were used as controls. Rats in the non-protein and methionine groups rather rapidly developed rough hair, showed inhibition of activity, and lost weight. The wounds of the non-protein group healed poorly. Although the animals in the methionine group had the general appearance of those in the non-

protein group, the wounds healed as rapidly and completely as those in the normal control group. The general appearance of rats in the valine group was somewhat better than in the non-protein and methionine groups but the wounds healed poorly.

A histologic and chemical study was made by Udupa, Woessner, and Dunphy (1956) on wound healing on normal diets, non-protein diets, and protein depleting diets supplemented with methionine. In rats maintained on a protein free diet, wound healing was retarded. The impairment of wound repair was correlated with a decreased accumulation of mucopolysaccharides and retarded collagen synthesis. Methionine added to the protein free diet restored the accumulation of mucopolysaccharides and the formation of new collagen fibers to levels which approximate the normal.

By the addition of methionine to a protein deficient diet, Williamson, McCarthy, and Fromm (1951) observed a correlation between protein sulfur retention and healing rate. Williamson and Fromm (1953) found that lysine, tryptophane, valine, and histidine had no significant effect on the rate of healing of experimental wounds when used to supplement a low or high protein diet, however, the administration of methionine to wounded or burned animals was shown to accelerate the rate of healing and to decrease the excretion of nitrogen. It was demonstrated by Williamson and Fromm (1953) that when protein depleted rats were injected with methionine containing labeled sulfur that the radio-sulfur was accumulated in the wound tissue even when the content of other tissues was decreasing.

Kieler (1953) studied the influence of various amino acids on mitotic activity in fibroblast cultures. A basic medium of dialyzed

material and tissue cultures of heart fibroblasts from eight day old chick embryos were used. Arginine, histidine, and lysine stimulated the mitotic activity of fibroblasts grown in the dialyzed medium. Glutamic acid and aspartic acid also promoted mitotic activity. Likewise, methionine and tryptophane were found to stimulate cell division. Cystine and proline were without definite effect. Later work by Kieler (1954) revealed that the mere presence of the necessary amino acids is not enough to secure normal mitosis. The relative concentrations of the various amino acids is equally important.

To determine the effect of the eight water soluble essential amino acids on the inflammatory reaction, Corcoran (1958) implanted these amino acids in the subcutaneous tissue of rabbits. Plugs of surgical cellulose sponge were saturated with solutions of the amino acids and placed under the skin of albino rabbits. The sponges were removed at 2, 7, 16, 23, 30, and 37 days. Lysine was the only amino acid to intensify the primary inflammatory response. It also stimulated fibroblastic proliferation and vascular tissue proliferation. Phenylalanine, arginine, and valine also produced an increased proliferation of fibroblasts accompanied by increased new vessel formation. Tryptophane markedly increased the cellularity and collagen production in the fibrous capsule surrounding the implants, however, fibroblastic invasion of the central spaces of the sponge was retarded. There was an absence of increased new capillary formation with the tryptophane implants. Histologically no significant differences were apparent between cellulose implants containing threonine, methionine, and histidine and the cellulose sponge control. Corcoran also investigated the effect of free crystals of amino acids placed in subcutaneous tissue and of crystals of amino acids packed in perforations

in lucite discs and implanted subcutaneously. His work indicated that similar tissue response does not occur to the same amino acid under different conditions of tissue exposure or concentration.

Certain preparations containing sulfhydryl are reported to stimulate wound healing when applied topically. Reimann (1930) found thiocresol to stimulate mitosis. Thioglycerol, a relatively stable sulfhydryl compound was successfully used by Sutton (1935) to stimulate wound repair in human patients. In over two hundred cases treated at the Mayo Clinic, Brunsting and Simonsen (1933) observed changes in the healing of cutaneous ulcers following the use of cysteine. There was stimulation of granulation tissue and epithelial proliferation and a clearing of secondary infection.

Macroscopic Aspects of Open Wound Healing

The healing of an open wound may be said to consist of four principal stages (Carrel 1910). The first stage is the quiescent period. This period extends from the time the wound is made to the beginning of granulous (proliferative) retraction. It varies from one day to four days. At the end of the quiescent period the edges of the wound begin to advance toward each other. This is the period of granulous (proliferative) retraction. The rate of repair is directly proportional to the size of the wound. This is followed by the period of epidermization in which epithelium spreads over the surface of the granulations. The length of this period is inversely proportional to the dimensions of the wound. The next stage, the period of cicatrization, is relatively long and completes the healing process.

Histologic Aspects of Wound Healing

The inflammatory reaction may be observed histologically by the subcutaneous implantation of inert material. With wounds of this type there is an absence of contraction, epidermization, and cicatrization. Bing (1955) recorded the tissue reaction to "Ivalon" (formalized polyvinyl alcohol) implanted subcutaneously in rats. Three days after implantation, fine fibrin threads and some leukocytes were seen in the meshes of the sponge. Nearly the same was observed in animals killed on the fifth day but the number of polymorphonuclear leukocytes was greater. A week after implantation, connective tissue was seen growing into the meshes from the surrounding subcutaneous tissue. At the same time a few foreign body giant cells were observed lying in close connection with the sponge. In sponges removed more than one month after implantation, the connective tissue reached the center of the sponge. The connective tissue contained a few giant cells and some mononuclear macrophages containing droplets. During this time the sponge was partly resorbed.

Edwards, Pernokas, and Dunphy (1957) reported similar findings with guinea pigs. Microscopic examination at six hours and at two days after surgery revealed moderately intense inflammatory reaction in the surrounding tissue. The interstices of the sponge contained numerous polymorphonuclear leukocytes scattered throughout amorphous granular and stringy material which stained pink with eosin. At four days fibroblasts had replaced inflammatory cells and were scattered throughout the sponge filling peripheral interstices entirely. At six days delicate collagen fibers began to appear particularly at the peripheral interstices

and were even more abundant at eight days. By twelve days the sponge had received its maximum deposit of collagen fibers. After thirty days is appeared to regress in fiber content.

Biochemical Aspects of Wound Healing

In a report of the biochemical aspects of fibrogenesis and wound healing, Jackson (1958) observed that the primary cellular reaction to the initial wound is concerned with preparation of the site for fibroblastic proliferation. Fibroblasts appear about the second day and proliferate rapidly. Before fibers are visible there is an increase in an amorphous material having the staining properties of acid mucopolysaccharide. There are some indications that mucopolysaccharides are precipitating agents necessary for formation of collagen fibers. After the formation of the amorphous matrix, collagen fibers appear first as thin wavy fibrils which increase in thickness and form typical mature collagen fibers. The increase in collagen concentration which occurs very rapidly between the fifth and fourteenth day is paralleled by an increase in tensile strength. Biochemically the fibroblast probably begins synthesizing the collagen precursor as early as the third day. The precursor is secreted into the extracellular space where it begins to aggregate into a fibrous structure. These fibrils gradually increase in thickness and become visible histologically.

Collagen has a unique amino acid composition with a high concentration of proline, hydroxyproline, and glycine and a low concentration of aromatic amino acids such as tyrosine (Eastoe, 1956). Stetten and Schoenheimer (1944) showed that proline is a precursor of hydroxyproline and that exogenous hydroxyproline is not utilized for collagen synthesis.

Williamson (1956) observed that some of the proteins which are synthesized in the wound tissue appear to contain appreciably more cystine and methionine than does either the normal skin tissue, the wound collagen, or procollagen. Using methionine- S^{35}, Williamson and Fromm (1953) found that the largest part of the S^{35} in the wound tissue appears as cystine-S^{35}. They concluded that the rate of healing is principally a function of the cystine content of wound tissue. Earlier (1952) they had found that methionine is converted to cystine before being used in the healing process but that some methionine per se was required. They further noted that the conversion of methionine to cystine was irreversible in vivo. Analysis of cystine from the hair of rats by Du Vigneaud, et al (1944) revealed that approximately 80% of its sulfur was derived from methionine. Localio, Morgan, and Hinton (1948) observed that the sulfhydryl radical was deficient and not readily available to the wound of the protein deficient rat and hypothesized that deficient sulfhydryl activity may be one of the reasons for delayed healing.

CHAPTER III

METHOD AND PROCEDURE

Materials

The animals used in this work were adult male white rats weighing approximately 400 to 450 Grams. These were obtained from the Holtzman Rat Company of Houston, Texas. At the beginning of the experiment, they were active, alert, and apparently free from disease.

The amino acids investigated were L-methionine, L-lysine (free base), and L-arginine (free base), supplied as dry crystals.[1] A concentrated solution of each of the amino acids was prepared by dissolving 240 mg. of amino acid in 8 ml. of distilled water.

Implants were made of Ivalon surgical sponge,[2] formulation M. Ivalon is a white polyvinyl alcohol sponge made by treating foamed polyvinyl alcohol with formaldehyde. The sponge was supplied in rectangular blocks, 6½ cm. x 13 cm. x 19 cm. Slices 5 mm. in thickness were made with a tissue knife and from these slices circular implants 9 mm. in diameter were cut by means of a number 4 cork cutter. The finished implants were placed in a test tube containing the amino acid solution and allowed to become completely saturated. Control sponges

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[1]Amino acids were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

[2]Ivalon is manufactured by Clay-Adams, Incorporated, New York City.

were placed in distilled water. It was calculated that each sponge absorbed approximately .4 ml. of solution containing 12 mg. of amino acid.

Experimental Procedure

Before beginning the experiment, all rats were maintained on a standard diet of Rockland rat diet (complete) developed by Rockland Farms, New York and manufactured by A. E. Staley Manufacturing Company, Decatur, Illinois. The average protein content of this diet was 21.93%.

Two weeks prior to implantation, one group of rats were placed on a protein depletion diet[1] and continued on this diet throughout the test period, a total of 56 days. The other group of rats was fed the standard Rockland diet during the entire experiment. A total of 31 rats received the protein depletion diet and 23 the standard diet. This number is exclusive of preliminary trials.

The Ivalon implants and all surgical instruments, with the exception of knife blades and suture needles were sterilized by autoclaving. Knife blades and suture needles were sterilized in a solution of alcoholic quarternary ammonium. The amino acid solutions were not sterilized; however, every effort was made to handle the material in a sterile manner during preparation.

Prior to surgery, a liberal area over the back and sides of each rat was clipped with a number 20 Oster blade. The back was then scrubbed with liquid soap and dried. After drying, alcohol was applied to the operative field.

The rats were anesthetized with ether and placed in ventral recumbency.

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[1]Diet was supplied by the Nutritional Biochemical Corporation, Cleveland, Ohio.

A longitudinal mid-dorsal incision approximately 15 mm. in length, through the skin, was made in the posterior thoracic region with a number 15 Bard-Parker blade. A subcutaneous pocket was prepared on each side by inserting a hemostat and spreading open the jaws. An implant was gently inserted into the subcutaneous pocket on each side with a flat surface down. One group received a control sponge on the left side and a methionine sponge on the right side while the other group received a lysine sponge on the left side and an arginine sponge on the right side. The implants were placed deep enough that they would be no closer than 1 cm. to the edges of the skin incision. Skin incisions were closed with two interrupted stitches of size 000 nylon dermal suture.

Implants were removed at 1, 3, 6, 9, 12, 18, 30, and 42 days following surgery. Rats were euthanized with intraperitoneal pentobarbital sodium and a liberal area of the overlying skin and adjacent subcutaneous tissue were removed along with the implant. After removal, the tissue was placed in individual capsules, identified, and fixed in 10% buffered formalin.

Plasma protein determinations were also made at 1, 3, 6, 9, 12, 18, 30, and 42 days. Blood samples were obtained from the heart with heparin being used as the anticoagulant. The specific gravity of the plasma was determined by means of copper sulfate solutions. The plasma protein was calculated as Grams per 100 ml.

In order to better evaluate the correlation between weight loss and protein depletion, the weights were recorded for one group of protein depleted and normal animals. They were weighed immediately following euthansia.

After fixation, the implant and adjacent tissue were bisected perpendicular to the skin and as exactly as possible through the center of the implant. The implants were embedded in paraffin with the cut surface down and histologic sections cut 5 microns in thickness. Sections from each block were stained with hematoxylin and eosin and Gomori's trichrome stain.

CHAPTER IV

RESULTS

Gross Observations

Experimental animals placed on the protein depletion diet steadily lost weight during the course of the experiment. Rats receiving the standard diet appeared to maintain their weight or gain slightly. The weights were recorded for one series of normal and protein depleted rats. The average weight of rats being fed a standard diet and receiving control implants was 429.2 Gm. (range 360 Gm. to 500 Gm.). Protein depleted rats receiving control and methionine implants had an average weight of 319.3 Gm. (range 250 Gm. to 370 Gm.) while those receiving lysine and arginine implants had an average weight of 320 Gm. (range 225 Gm. to 355 Gm.). Figure 1 shows a protein depleted rat in the foreground and a normal rat behind. The photograph was made forty-two days after surgery (56 days after beginning the experiment).

Rats fed the protein depletion diet remained alert but were less active than those receiving the standard diet. There was no difference in behavior of rats receiving control and methionine implants and those receiving lysine and arginine implants. Consumption of the protein depletion diet was poor and considerable wastage was observed.

Hair growth was poor in rats receiving the protein depletion diet. In the area that was clipped in preparation for surgery, replacement of hair was slight even by forty-two days following surgery in the protein

Fig. 1. Comparison of normal and protein depleted rats. Protein depleted rat is in front.

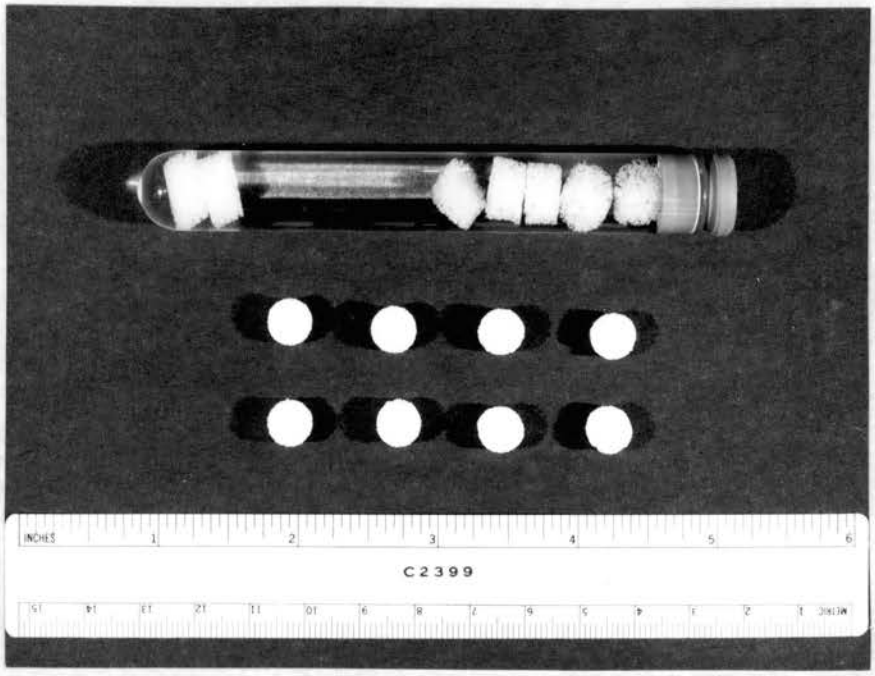

Fig. 2. Cylinders of ivalon surgical sponge (wet and dry specimens).

depleted groups. Hair was rapidly replaced in the animals receiving a standard diet (Figure 1). Several of the protein depleted rats developed a generalized alopecia of moderate severity that was first evident about thirty days after beginning the experiment.

Surgical incisions in the mid-dorsal thoracic region healed more slowly in the protein depleted rats than in the normal controls. This was evident at necropsy by gaping of wounds when the sutures were removed. There was a much greater tendency for the protein depleted rats to molest the incision of other rats in the group than occurred with the normal groups. Disturbing the incision lead to removal of the sutures in some individuals. In several cases, the skin incisions became opened in the protein depleted rats necessitating the isolation of these rats. There were four cases of wound dehiscence in the protein depleted groups receiving control and methionine implants and eleven cases in protein depleted groups receiving lysine and arginine implants. The wounds became disrupted between the second and fourth postoperative day in the control and methionine groups and between the first and eighth day in the lysine and arginine groups. No cases of wound dehiscence occurred in rats receiving the standard diet.

Two deaths occurred in rats during the course of the experiment. One death occurred in a normal control on the fifteenth day after implanting and one in a protein depleted rat on the forty-second day after implanting. No illness was noted in other rats described in this investigation.

Necropsies of the protein depleted rats revealed a marked decrease in body fat. However, there was no evidence of anasarca, ascites, hydrothorax, or other indications of a hypoproteinemia. All internal organs

appeared essentially normal.

The plasma protein level was determined for one series of rats. The average value for normal rats receiving control implants was 6.21 Gm. per 100 ml. (range 5.81 to 6.84 Gm. per 100 ml.). For the protein depleted rats, the average value was 5.13 Gm. per 100 ml. (range 4.78 to 5.48 Gm. per 100 ml.) in those receiving control and methionine implants and 4.95 Gm. per 100 ml. (range 4.45 to 5.48 Gm. per 100 ml.) in those receiving lysine and arginine implants.

Histologic Findings

The observations of ninety-two implants were used in compiling this report. Four implants were not included in the data because of illness of the animal. Examination of these four implants revealed that coincident disease resulted in marked retardation of fibroplasia in these instances.

Control Implants in Rats on a Standard Diet

The most striking feature of the one day implants was the presence of many polymorphonuclear leukocytes throughout the interstices of the sponges. Pale pink stringy material believed to be fibrin was observed within the sponge spaces particularly at the periphery. There was a moderately intense inflammatory reaction in the subcutaneous tissue surrounding the implants with hyperemia and increased cellularity. Predominant cell types present were polymorphonuclear leukocytes, macrophages, and fibroblasts. Distinct basophilic staining of a narrow zone in the subcutaneous tissue immediately overlying the implants was observed. This was thought to be a degenerative change produced by pressure

from the implants. In this zone the collagen fibers appeared compressed.

At three days polymorphonuclear leukocytes continued to be numerous. The pinkish stringy material was still present within the sponges. The inflammatory reaction in the surrounding connective tissue had persisted. There was a faint zone of compressed collagen around the implants.

In the six day implants, large fibroblasts had penetrated the peripheral interstices of the sponge (see Figure 3) and collagen deposition could be visualized in the sections receiving Gomori's trichrome stain. Beginning proliferation of small capillaries was noted. There was a marked reduction in the number of polymorphonuclear leukocytes. Some macrophages were found within the sponges. The acute inflammatory reaction in the surrounding subcutaneous tissue had decreased in intensity.

At nine days fibroblast proliferation had penetrated more deeply into the interstices of the sponges (see Figure 5). The fibroblasts were arranged as bundles or finger like projections within the interstices of the sponges. The newly formed tissue was quite vascular and contained delicate collagen fibers. In the central portion of the sponge, macrophages were seen lying in the strands of fibrin.

Very rapid fibroblast proliferation had taken place between the ninth and twelfth day (see Figure 7). After twelve days loose connective tissue filled the sponge meshes throughout the implant. There was a corresponding increase in collagen production. A few foreign body giant cells were distributed throughout the implants.

In the eighteen day implants, fibroblasts within the sponge meshes had become more flattened and elongated and collagen was slightly more abundant. The implants were seen to be thicker than at twelve days. There was a slight increase in the number of foreign body giant cells.

After thirty days the fibrous connective tissue had increased slightly in amount beyond what was observed in the eighteen day implants and had become somewhat less cellular. The collagen fibers were broader than in the preceeding implant, nearly filling the sponge spaces.

Little if any difference could be detected between the thirty and forty-two day implants. Apparently the maximum deposition of collagen had occurred within thirty days.

Effect of Amino Acids in Rats on a Standard Diet

In Tables I and II the inflammatory response to control implants are compared to the response observed in implants saturated with methionine, lysine, and arginine. Only at nine days could any significant difference be detected. At this stage, fibroblast proliferation had extended more deeply into the central area of the sponge in the methionine implants than it did in the control implants (see Figures 5 and 6). There was also a greater amount of collagen in the methionine implants. A similar difference was observed in preliminary studies. The lysine and arginine implants exhibited less extensive fibroblast proliferation than did the methionine implants. They appeared essentially the same as control implants throughout the course of the experiment. No difference could be detected in control implants placed in animals with methionine implants and control implants placed in animals without methionine implants.

Control Implants in Protein Depleted Rats

At one day numerous polymorphonuclear leukocytes had invaded the sponges. The leukocytes appeared to be oriented along strands of fibrin.

TABLE I

INFLAMMATORY RESPONSE TO SUBCUTANEOUS SPONGE IMPLANTS IN NORMAL RATS, 3 TO 9 DAYS

Days	Amino Acid	Response
3	Control	Numerous polymorphonuclear leukocytes had infiltrated into the sponges. Pink stringy to granular material was found within the sponge spaces. A moderately intense inflammatory reaction was observed in the surrounding tissue.
	Methionine	No significant change from control.
	Lysine	No significant change from control.
	Arginine	No significant change from control.
6	Control	Fibroblasts had invaded the peripheral interstices of the sponges. Beginning collagen deposition was present at the periphery. There was a marked reduction in the number of polymorphonuclear leukocytes. The acute inflammatory reaction in the surrounding subacutaneous tissue was less intense than at 3 days.
	Methionine	No significant change from control.
	Lysine	No significant change from control.
	Arginine	No significant change from control.
9	Control	The fibroblast proliferation extended more deeply into the interstices of the sponges than at 6 days. Collagen fibers were visible in the newly formed connective tissue. Proliferating capillaries were numerous.
	Methionine	Fibroblast proliferation penetrated more deeply into the central area of the sponge than in the control implants. There was a corresponding increase in collagen production.
	Lysine	No significant change from the control.
	Arginine	No significant change from the control.

TABLE II

INFLAMMATORY RESPONSE TO SUBCUTANEOUS SPONGE IMPLANTS IN NORMAL RATS, 12 TO 42 DAYS

Days	Amino Acid	Response
12	Control	Proliferating fibroblasts filled the sponge meshes throughout the implant. There was a corresponding increase in collagen production.
	Methionine	No significant change from control.
	Lysine	No significant change from control.
	Arginine	No significant change from control.
18	Control	Fibroblasts within the sponge meshes had become flattened and more elongated. Collagen was slightly more abundant than at 12 days.
	Methionine	No significant change from control.
	Lysine	No significant change from control.
	Arginine	No significant change from control.
30	Control	Fibrous connective tissue had become slightly more dense than was observed in the 18 day implants. Collagen was more abundant and the fibers broader.
	Methionine	No significant change from control.
	Lysine	No significant change from control.
	Arginine	No significant change from control.
42	Control	It appeared essentially the same as 30 day implants.
	Methionine	No significant change from control.
	Lysine	No significant change from control.
	Arginine	No significant change from control.

Blood vessels in the surrounding subcutaneous tissue were dilated and polymorphonuclear leukocytes were seen near the implant. The tissue immediately adjacent to the implant appeared compressed and in some areas the tissue took a basophilic stain.

Polymorphonuclear leukocytes continued to be numerous at three days. The inflammatory reaction in the surrounding tissue was quite similar to that seen at one day.

In the six day implants, the number of polymorphonuclear leukocytes was less than was observed at three days. The nuclei of many of the leukocytes had undergone pyknosis or karyorrhexis. An occasional fibroblast was visible at the periphery of the implant (see Figure 4).

By nine days scattered fibroblasts were present at the periphery of the implants. In this area very faint staining material resembling collagen was visible when stained with Gomori's trichrome stain. Beginning capillary proliferation was seen in the areas of fibroblast invasion. Neutrophils and mononuclear cells were distributed throughout the sponge in moderate numbers. Most of these cells had undergone degenerative changes.

At twelve days fibroblast proliferation had penetrated more deeply toward the center of the sponges (see Figure 8). There was a corresponding increase in collagen production. An occasional foreign body giant cell was present at this time.

After eighteen days, the proliferating fibroblasts filled most of the sponge spaces. Collagen was found throughout the areas of fibroblast proliferation, however, the collagen appeared as widely separated, thin, wavy fibrils.

Proliferating fibrous connective tissue completely filled the sponge

spaces throughout the entire implant by thirty days. Some of the fibroblasts had become more flattened and elongated. There was no detectable increase in the amount of collagen. A moderate number of mononuclear cells and several large plump fibroblasts were seen within the more mature fibrous tissue in the implant.

At forty-two days plump fibroblasts continued to be present. No increase in collagen could be detected.

Effect of Amino Acids on Protein Depleted Animals

Considerable individual variation in inflammatory reaction was encountered in the protein depleted rats. Consistent differences that might be attributed to any particular amino acid were slight. The response to methionine, lysine, and arginine are compared to controls in Tables III, IV, and V. It appeared that there was a slightly greater fibroblast invasion with methionine implants at six and nine days than with control implants. It must be emphasized, however, that the difference was slight and not entirely conclusive. There was no indication that collagen production was stimulated by any of the amino acids tested.

Comparison of Inflammatory Response in Normal and Protein Depleted Rats

At one day, numerous polymorphonuclear leukocytes had invaded the implants of both the protein depleted and normal rats. Moderate hyperemia and cellular infiltration of neutrophils and mononuclear cells were observed in the surrounding subcutaneous tissue in both groups.

After three days, polymorphonuclear leukocytes continued to be found in large numbers within all implants. The acute inflammatory reaction in the surrounding subcutaneous tissue persisted in both the protein

TABLE III

INFLAMMATORY RESPONSE TO SUBCUTANEOUS SPONGE IMPLANTS IN PROTEIN DEPLETED RATS, 1 TO 6 DAYS

Days	Amino Acid	Response
1	Control	Numerous polymorphonuclear leukocytes were lying in strands of pink granular to stringy material within the sponge spaces. There was hyperemia and cellular infiltration of the surrounding subcutaneous tissue.
	Methionine	No significant change from control.
	Lysine	No significant change from control.
	Arginine	No significant change from control.
3	Control	Polymorphonuclear leukocytes continued to be numerous. Very little difference between this and the one day implants was detected.
	Methionine	No significant change from control.
	Lysine	No significant change from control.
	Arginine	No significant change from control.
6	Control	Marked reduction in the number of polymorphonuclear cells was observed. A small number of fibroblasts were visible at the periphery of the implant.
	Methionine	A slightly greater number of fibroblasts were detected at the periphery of the implant.
	Lysine	No significant difference from control.
	Arginine	No significant difference from control.

TABLE IV

INFLAMMATORY RESPONSE TO SUBCUTANEOUS SPONGE IMPLANTS
IN PROTEIN DEPLETED RATS, 9 TO 18 DAYS

Days	Amino Acid	Response
9	Control	Areas of fibroblast invasion were present at the periphery of the implants. Very faint staining material resembling collagen was visible when stained with Gomori's trichrome stain.
	Methionine	There appeared to be a slightly greater number of fibroblasts than was present in control implants.
	Lysine	No significant difference from control.
	Arginine	No significant difference from control.
12	Control	Fairly rapid growth of fibroblasts occurred between 9 and 12 days. There was a corresponding increase in amount of collagen. Collagen fibers were delicate and widely separated.
	Methionine	No significant change from control.
	Lysine	No significant change from control.
	Arginine	No significant change from control.
18	Control	Fibroblast proliferation extended throughout most of sponge leaving only a small central area unfilled. At the periphery collagen was slightly more abundant and fibers thicker than at 12 days.
	Methionine	No significant change from control.
	Lysine	No significant change from control.
	Arginine	No significant change from control.

TABLE V

INFLAMMATORY RESPONSE TO SUBCUTANEOUS SPONGE IMPLANTS IN PROTEIN DEPLETED RATS, 30 TO 42 DAYS

Days	Amino Acid	Response
30	Control	Proliferating fibrous connective tissue filled the entire implant. There was little if any increase in amount of collagen.
	Methionine	No significant change from control.
	Lysine	No significant change from control.
	Arginine	No significant change from control.
42	Control	No increase in collagen content was detected.
	Methionine	No significant change from control.
	Lysine	No significant change from control.
	Arginine	No significant change from control.

depleted and normal animals.

The first indication of a difference in the inflammatory response of protein depleted and normal rats was seen at six days (see Figures 3 and 4). At this time, there were definite areas of fibroblast proliferation at the periphery of implants in the normal rats while in the protein depleted rats only a few fibroblasts were seen at the periphery of the implant. Beginning collagen deposition was observed in the areas of proliferating fibroblasts in the normal animals, however no trace of collagen was seen in the protein depleted animals.

In nine days, the fibroblast proliferation had extended more deeply into the implants of normal rats and there was a corresponding increase in collagen deposition. With the protein depleted rats, the degree of fibroplasia at nine days was somewhat less than that observed in the normal rats at six days and the fibroblasts appeared as loosely arranged groups of cells. In normal animals fibroblasts grew as compact bundles within the sponge spaces.

Rapid proliferation of fibroblasts occurred in both protein depleted and normal rats between nine and twelve days (see Figures 7 and 8). Fibroblasts filled the sponge meshes throughout the implants of the normal rats, however, the central portion of the implants was not filled in the protein depleted rats. Collagen was more abundant and the fibers broader in the normal rats than in the protein depleted rats.

Even at eighteen days the central portion of the sponges of the protein depleted rats was not completely filled. The implants of the normal control animals continued to contain more collagen.

There was a marked difference in collagen deposition at thirty days. In the normal animals the collagen was dense, nearly filling the

sponge spaces. In the protein depleted animals the collagen was present as wavy, delicate, widely separated fibers quite similar to the twelve day implants in the normal animals.

The most noticeable difference between the forty-two day implants was the much greater thickness of the implants from the normal animals (see Figures 9 and 10). This difference could be detected by gross observation. On microscopic examination it was apparent that collagen was much more abundant in the normal animals. In the protein depleted rats the collagen fibers continued to be relatively thin and widely separated.

Fig. 3. Control implant from normal rat at six days. H & E stain; xl2

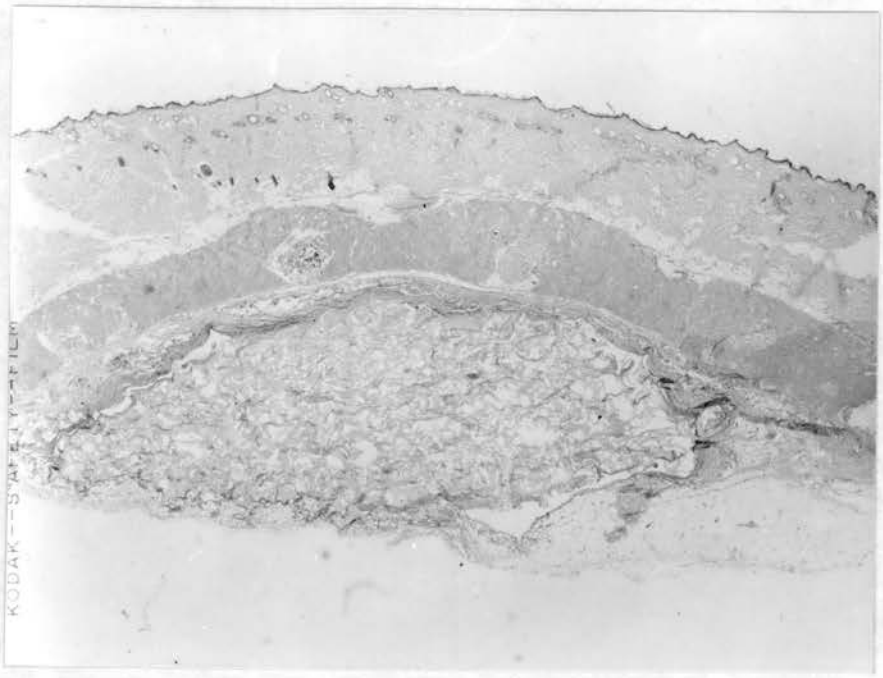

Fig. 4. Control implant from protein depleted rat at six days. H & E stain; xl2.

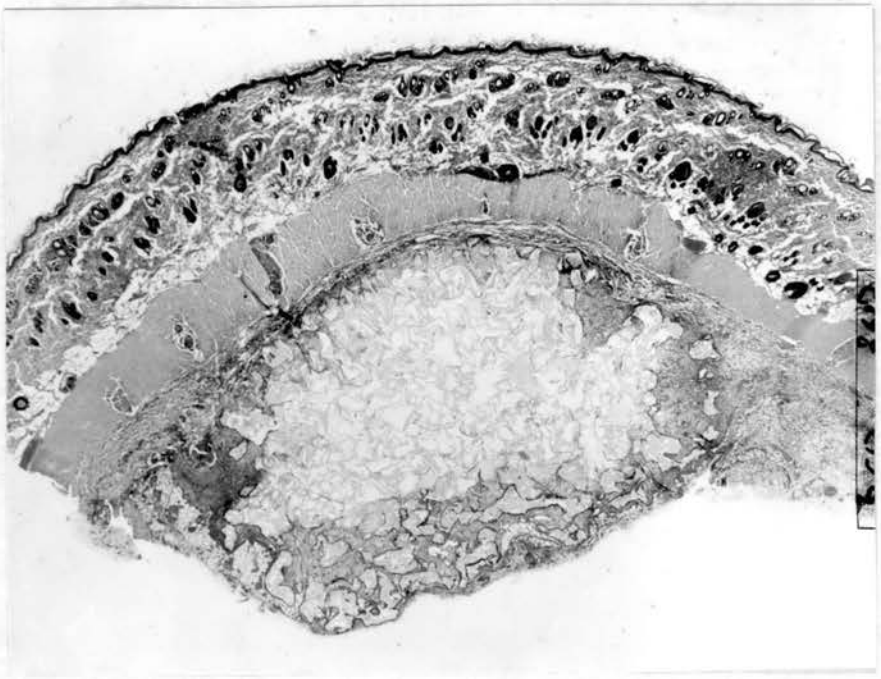

Fig. 5. Control implant from normal rat at nine days. H & E stain; xl2.

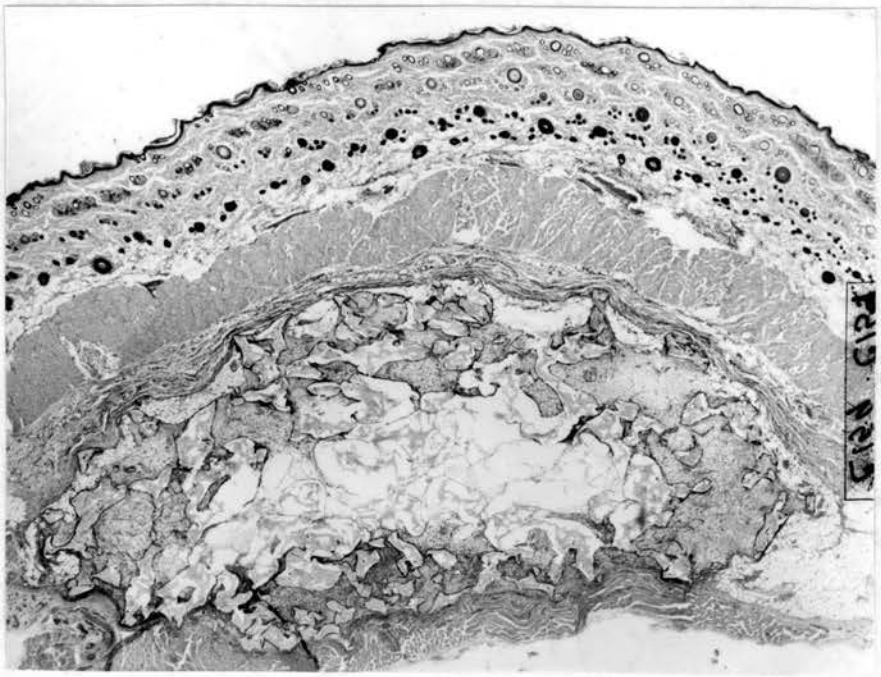

Fig. 6. Methionine implant from normal rat at nine days. H & E stain; xl2.

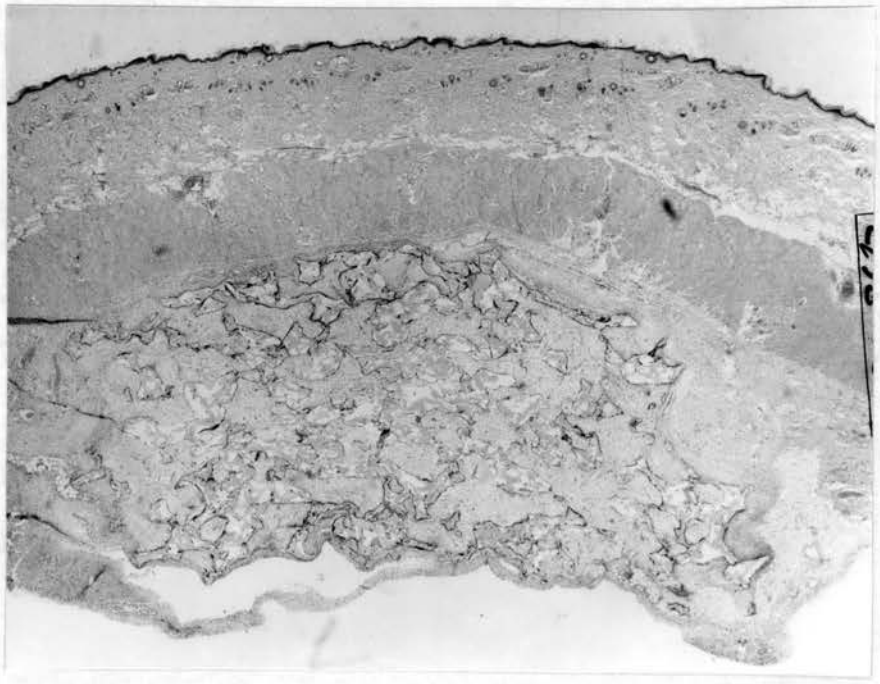

Fig. 7. Control implant from normal rat at twelve days. H & E stain; x12.

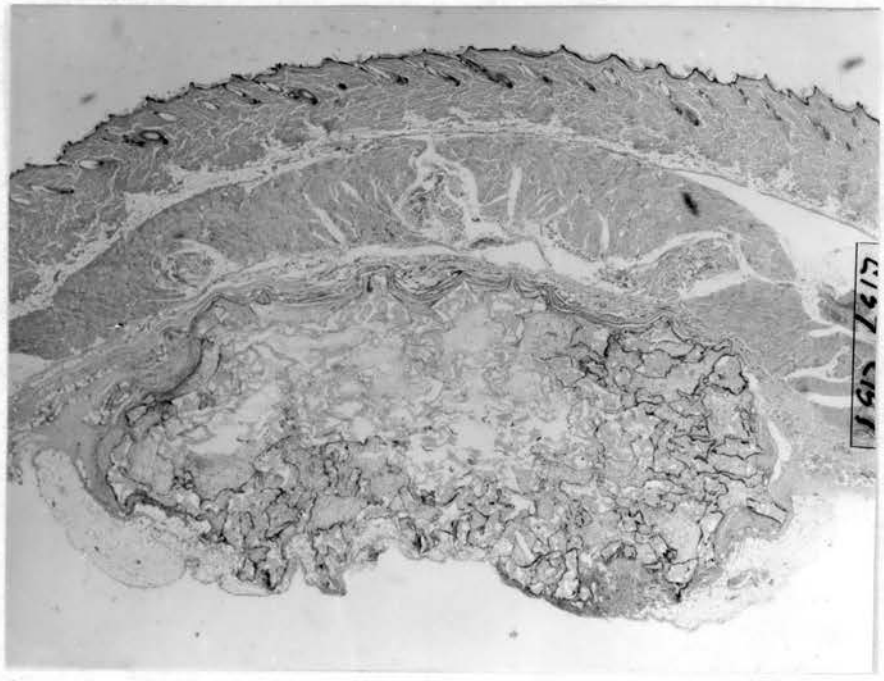

Fig. 8. Control implant from protein depleted rat at twelve days. H & E stain; x12.

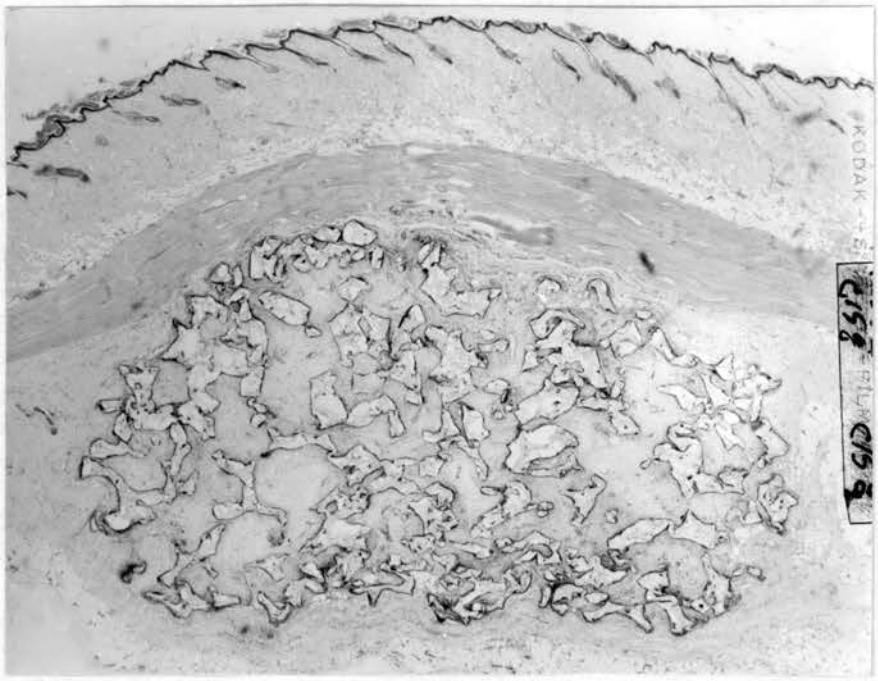

Fig. 9. Control implant from normal rat at forty-two days. H & E stain; xl2.

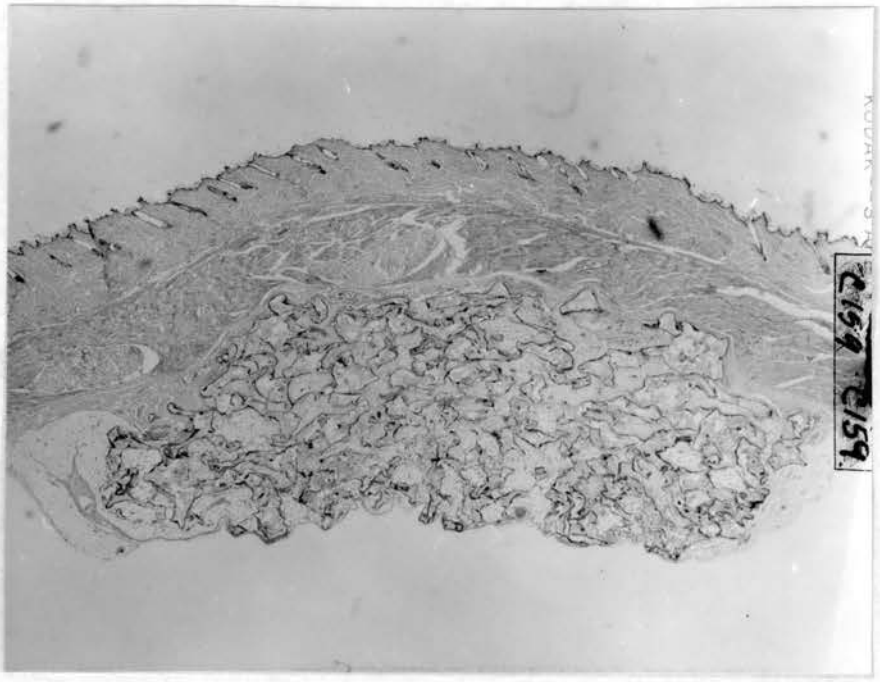

Fig. 10. Control implant from protein depleted rat at forty-two days. H & E stain; xl2.

CHAPTER V

DISCUSSION AND CONCLUSIONS

There were three principal objectives in this investigation. They were as follows:

1. To devise a method of accurately studying the influence of chemical substances on subcutaneous inflammatory reactions.
2. To measure the difference in healing rate between animals on a standard diet and those on a protein depletion diet.
3. To determine the effect of certain water soluble amino acids on the inflammatory reaction in normal animals and in protein depleted animals.

Various methods and materials were employed in the preliminary work in an effort to devise a means of studying the inflammatory reaction. The technique found most satisfactory consisted of the subcutaneous implantation of cylinders of formalized polyvinyl sponge in white rats. Test sponges were saturated with solutions of the amino acids to be studied which were methionine, lysine, and arginine. Control sponges were saturated with distilled water.

By this method vascular changes, exudative reaction, fibroblast proliferation, and collagen deposition could be observed and compared with controls. It was vital that the implants always be cut at the same plane and exactly through the center for valid comparisons to be made. Contraction of the skin margins which accounts for the rapid closure of external wounds was eliminated by the use of subcutaneous implants.

The subcutaneous implants were used to compare the inflammatory reaction in protein depleted and normal animals. In order to produce protein depletion, rats were placed on a protein free diet for a period of two weeks before beginning the experiment and continued on this diet throughout the entire experimental period. This was considered the minimum amount of time required for depletion of normal protein reserves in the body. The plasma protein levels were consistently lower than in animals receiving a standard diet but at no time did they reach extremely low levels. None of the lesions commonly associated with hypoproteinemia were encountered during the experiment. Apparently the body is able to maintain the plasma protein at near normal levels despite severe depletion of tissue protein.

No difference could be detected between normal and protein depleted rats in the initial inflammatory reaction. However, beginning at six days after surgery a decided difference was noted in these two groups. Protein depletion caused a delay in the onset of fibroplasia, decreased proliferation of fibroblasts, and retarded collagen deposition.

It has been proposed that the relation of protein deficiency to wound healing is mainly on an osmotic basis and that fibroplasia was hindered by the excessive amounts of tissue fluid. This investigation would tend to refute the singularity of that theory. There was at no time any evidence of excessive tissue fluids yet fibroplasia was inhibited when tissue protein had been depleted. This would seem to indicate that the relation of protein deficiency to wound healing is associated with the availability of essential nutrients to individual cells.

The subcutaneous inflammatory response to methionine, lysine, and arginine were compared to controls in protein depleted rats and in rats

on a standard diet. Considerable study has been made by previous investigators on the influence of these amino acids when administered parenterally but no published records were found of the results achieved by placing this material directly in the subcutaneous tissue of protein depleted rats.

None of the amino acids tested had any detectable influence on the early vascular and exudative changes in either of the protein depleted or normal animals. However, at nine days after implanting the sponges, there was greater proliferation of fibroblasts and deposition of collagen in normal rats receiving methionine implants than in normal rats receiving control implants. Similar results were obtained in preliminary work. There was slightly greater fibroblast proliferation in protein depleted rats receiving methionine implants at six and nine days after implanting than in protein depleted rats receiving control implants. This difference was slight. Lysine and arginine apparently had no influence on the inflammatory response in either protein depleted rats or rats receiving a standard diet.

The reason for a stimulation of fibroplasia by methionine in animals presumably receiving their normal requirement of protein is not clearly understood. Previous work has indicated that the topical application of various compounds containing sulfhydryl promotes wound healing. Furthermore, there are reports of more rapid healing in animals on a high protein diet than in animals on only an adequate diet. Perhaps this is due to a more abundant supply of methionine.

Some reports indicated that methionine exerted a definite stimulating effect on fibroplasia in protein depleted rats. The influence of methionine was ascribed to the activity of the sulfhydryl group.

The lack of more dramatic results with methionine in this investigation is attributed at least in part to the small amount that could be introduced into subcutaneous tissue. The fact that any difference could be detected with such a small amount of methionine appears significant. The amount was limited by the solubility of the amino acid and the size of the implant. Implants of the size used would absorb a maximum of 0.4 ml. of the solution containing 30 mg. of amino acid per ml. This means that each implant contained not more than 12 mg. of amino acid. In previous work methionine had been administered to protein depleted rats at the rate of approximately 20 mg. per day in the diet throughout the experiment or injected subcutaneously at the rate of 150 mg. per day for 10 days following surgery. For further work in this area a method should be devised by which a greater concentration of amino acid could be introduced into the wound.

CHAPTER VI

SUMMARY

An investigation of the subcutaneous inflammatory response to certain water soluble amino acids in Ivalon sponges was conducted. Methionine, lysine, and arginine preparations were made by saturation of the sponges with solutions of these substances prior to implanting. The study was made with both normal and protein depleted rats.

The early vascular and exudative changes seen in the one and three day implants were essentially the same in the protein depleted and normal animals. Similarly, no alteration of this phase of inflammation by any of the amino acids was detected.

Beginning at six days after surgery a definite differences were apparent in the inflammatory response of the protein depleted and normal animals. These differences continued for the duration of the experiment (42 days). Protein depletion caused a delay in the onset of fibroplasia, decreased poliferation of fibroblasts, and retarded collagen deposition.

Changes that could be attributed to any of the amino acids were seen only during the early stages of fibroblast proliferation. At nine days after implanting the sponges, there was a greater proliferation of fibroblasts and deposition of collagen in normal rats receiving methionine implants than in normal rats receiving control implants. There was slightly greater fibroblast proliferation in protein depleted rats receiving methionine implants at six and nine days after implanting

than in protein depleted rats receiving control implants. Lysine and arginine apparently had no influence on the inflammatory response in either protein depleted rats or rats receiving a standard diet.

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Master of Science

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