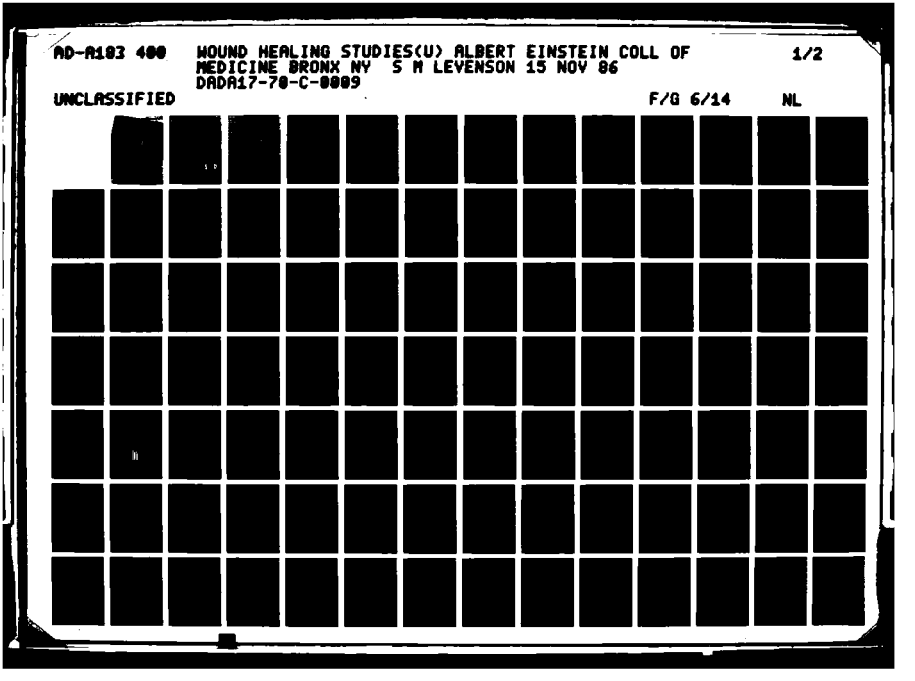
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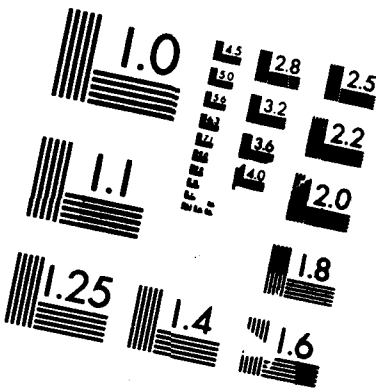

WOUND HEALING STUDIES

ANNUAL/FINAL REPORT

STANLEY M. LEVENSON, M.D.

(FOR THE PERIOD AUGUST 1, 1969 - MARCH 15, 1984

15 NOVEMBER 1986

SUPPORTED BY

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SUMMARY

Studies directed toward improving wound healing and resistance to wound infection of severely injured soldiers have been carried out aimed at establishing improved prophylactic and therapeutic measures. The studies are based on our view that some of the complex series of events which underlie the reparative process and resistance to wound infection are affected adversely by injury and that these may be modified by nutritional, chemical, and hormonal means, singly and in combination, in ways to improve the rate and quality of wound healing, increase resistance to infection and thereby shorten convalescence and speed the return to active duty.

Emphasis has been placed on modification of the inflammatory reaction, reparative vessel formation, fibroblastic proliferation, collagen synthesis, collagen cross-linking and collagen resorption, employing in vitro and in vivo studies, including the effects of supplemental vitamin A, beta carotene, arginine and ornithine. Studies to develop methods to ameliorate the adverse effects of trauma and radiation on wound healing, wound infection, gastrointestinal ulceration, and survival have also been conducted as well as the effects of combined thermal and radiation injury. Studies also have been carried out on how certain bacteria influence wound healing based on our observation that certain strains of Staphylococcus aureus accelerate remarkably the rate of gain of strength of healing incisions. Studies of liver regeneration have also been carried out since we believe that investigation of hormonal factors which regulate and modulate liver regeneration will cast light on the questions of why a wound begins to heal, what keeps it going and what stops it. Also, studies have been conducted with a new hemostatic scalpel; these were undertaken because there are a number of surgical operative procedures where the attendant blood loss is a serious threat to the patients; early excision of extensive deep burns is a notable example of this. Studies of the chemical debridement of burns were also conducted.

The resutls of these various investigations, which are described in the body of the report, offer the potential of providing significant improvement in the care of injured soldiers.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission of Life Sciences, National Research Council (NIH Publication No. 86-23, Revised 1985).

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ABSTRACTS

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INTRODUCTION

This Final Progress Report includes our Annual Progress Report for the period 1 November 1983 - 15 March 1984.

As stated in our previous Progress Reports, the last dated 1 November 1983, a central aim of our studies is the improvement of wound healing of severely injured soldiers. To this end, our studies are directed towards an increased understanding of the basic biochemical, physiologic, and morphologic factors involved in healing and how these are affected by serious injury. Such knowledge is fundamental to establishing improved prophylactic and therapeutic measures.

We have mentioned in our previous reports that wound healing may be considered as a specific biological process related to the general phenomenon of growth and regeneration. The various states (including inflammation, blood vessel and lymphatic formation, fibroblastic proliferation, proteoglycan and collagen synthesis, collagen cross-linking, collagen resorption, remodelling of the wound) indicate that the entire process normally is an orderly one, showing a high degree of integration and organization characteristic of processes in which control mechanisms are operative. These periods are not sharply separated. Factors which stimulate or inhibit one phase of the process have an effect on the overall process, the magnitude of the effect depending in part on how "rate-limiting" is the facet of healing being affected.

Our investigations have been based on our view that some of the key steps in the highly integrated processes of wound healing are affected adversely by serious trauma and that these may be modified in ways to improve the rate and quality of wound healing and thereby shorten convalescence. We have also carried out some studies to attempt to ameliorate the response to radiation injury and radiomimetic drugs. Our approaches have included the use of nutritional, chemical and hormonal means. Since wound healing and wound infection are so closely interrelated, studies of wound infection have also been carried out. We have pursued our serendipitious observation that purposeful contamination of skin incisions of rats immediately after wounding with certain strains of Staphylococcus aureus leads to a dramatic acceleration in the rate of gain of wound strength, much greater than has hitherto been described for any agent or maneuver. Studies of liver regeneration and chemical and surgical debridement of burns, the latter with a new hemostatic scalpel were also conducted.

1. Attempts to ameliorate the adverse effects of serious injury on the metabolic reaction to injury, on wound healing and host resistance by various nutritional, chemical, hormonal and environmental means

There are considerable data regarding the multiple interrelated metabolic changes which follow serious injury but much less data as to how these affect physiologic and clinical responses. To elaborate: During the past 50 years there have been many studies showing that following injury changes occur involving energy, protein, carbohydrate, fat, water, vitamin, and mineral metabolism. Underlying these is a variety of factors, including alterations in neuro-endocrine activity; interactions among the components of the neuro-endocrine system are of major significance. There are also data indicating that release of interleukin 1 (leukocytic endogenous pyrogen (LEM)) and/or one of its breakdown products (e.g., a peptide of 4274 daltons) plays a key role, including stimulation of skeletal muscle proteolysis. The responses are dynamic, parallel the severity of the injury and follow a generally predictable pattern but are still incompletely understood. These will not be detailed here since they are reviewed in many publications.

A multiplicity of factors modify these metabolic changes, including age, sex and prior nutritional status of the patients, nature and severity of the injury, nutrient intake, immobilization, and environmental temperature and relative humidity. These metabolic changes have profound effects on the injured individual's nutrition, wound healing (established in experimental animals), and defense mechanisms.

Our understanding of these matters is still incomplete and we do not fully understand the mechanism underlying these metabolic changes, nor do we fully understand their physiologic consequences. We will not discuss these matters in detail in this short section. We will focus in this discussion on the questions: How do these metabolic changes affect the clinical course and convalescence of the injured patients particularly from the points of view of wound healing? Should attempts be made to modify them.

Systematic studies of the healing of operative and traumatic wounds should provide objective evidence in one important area as to the significance of the early metabolic derangements. Although some have inferred that wound healing is "normal" in the early period after injury, the evidence with experimental animals is clear: wound healing is seriously impaired after injury.

Thus, we showed a number of years ago at the Army Medical Nutrition Laboratory and at the Walter Reed Army Institute of Research that the healing of laparotomy wounds made in rats (Surg., Gynecol. Obstet. 99: 74 (1954)) and guinea pigs (Ann.Surg. 146: 357 (1957)) one day after they were severely burned on their backs was significantly slower than in unburned controls. This was independent of any possible adverse effect of shock per se since in each of the studies the animals had been adequately treated from the start with saline and had never been in shock.

A. Effect of Femoral Fracture

More recently, we have shown that the healing of dorsal skin incisions and the formation of reparative granulation tissue in response to subcutaneously implanted polyvinyl alcohol sponges is impaired in rats subjected to unilateral or bilateral comminuted femoral fractures. We turned to this experimental model for our further studies of the effect of injury on wound healing because (1) studies in our laboratory over a period of years have shown that dorsal skin incisions and implanted polyvinyl alcohol sponges have certain advantages over laparotomy wounds; and (2) femoral fractures have some advantage over burns - the water vapor loss through the burned area and presence of bacteria with infection of varying severety introduce certain variables which complicate the study and its interpretation. The impairment in healing was somewhat greater in the rats subjected to bilateral femoral fracture than in those with unilateral fracture as judged by the formation of reparative collagen.

B. Effect of Testosterone Propionate

In related experiments, we showed that the administration of testosterone propionate (s.c. or i.m.) to rats had no influence on wound healing of rats with or without femoral fracture. We conducted these experiments because earlier work by ourselves (Proc. Soc. Exp. Biol. & Med. 75:183(1950)) and others, e.g., Abbott and Hirschfeld (J.Clin. Invest. 26:796(1947)) had shown that the increased urinary nitrogen excretion which characteristically follows injury (and the intensity of which is proportional to the severity of the injury) is lessened by the administration of testosterone propionate to experimental animals and patients. However, there had been no evidence whether this was of any physiologic or clinical benefit. Our data indicate that it is not from the point of wound healing - that is, the impaired healing of rats with femoral fracture was not improved at all by administration of testosterone beginning on the day of wounding and fracture.

C. Effect of Environmental Temperature

Caldwell and his associates (Ann.Surg. 155:119(1962); (Surgery 150: 976,

(1959)) and Cuthbertson and his associates (Quart J. Exp. Physiol. 52, 114 (1967)) (Brit. J. Surg., 55, 513 (1968)) have reported that the increased urinary nitrogen excretion of rats with burns (Caldwell) or femoral fracture (Cuthbertson) is sharply reduced when they are adapted to a higher than normal ambient temperature (30° C vs. 22° C) prior to injury and maintained at the higher temperature. Cuthbertson also reported a limited experiment which suggested that this was also so when rats maintained at 22° C prior to injury were transferred to 30° C immediately after injury. Our own first experiments reported in the March 1971 progress report showed that when rats in a 22° C room with or without femoral fracture were transferred just after injury or sham-injury to a 30° C room, there was a marked drop in food intake immediately, and a significant reduction in food intake persisted during the 6-day post injury period of study. In this experiment, the rats at 30° C without fracture were pair-fed against the rats at 30° C with fracture and similarly, the rats at 22° C without fracture were pair-fed against the rats with fracture at 22° C. The latter two groups of rats had higher food intakes than the former two groups. Urinary nitrogen excretion was similar for the fractured and sham-fractured rats at each of the environmental temperatures, but the ratios of urinary nitrogen:nitrogen intake were substantially greater for the rats at 30° C, a reflection, we believe, of their markedly reduced food intake. The breaking strength of skin wounds of the rats with fracture at 30° C was lower than that of their sham-fractured controls at 30° C when tested in the fresh state, but not after formalin fixation, nor were there any differences in the hydroxyproline controls of the sponge granulomas in these two groups.

D. Effects of the Combination of Testosterone Propionate and Environmental Temperature

Experiments were conducted to determine whether the combination of higher environmental temperature (30° C) and the administration of testosterone propionate would have an "additive" effect on (1) urinary nitrogen, potassium and sodium excretions after injury and (2) the healing of dorsal skin incisions and the formation of granulomas in response to s.c. implanted polyvinyl alcohol sponges in rats with unilateral femoral fracture as compared with sham-fractured control rats.

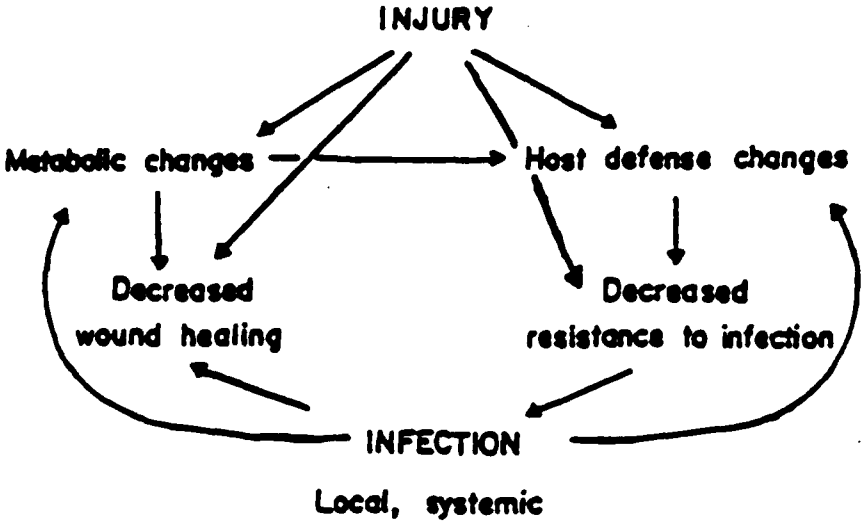
Our experiments have shown that when rats in a 22 °C room with or without femoral fracture were transferred just after injury or sham-injury to a 30 °C room, there was a marked drop in food intake immediately. Accordingly, we have conducted both pair-feeding and ad libitum feeding experiments. Among pair-fed rats without fracture, wound healing was little different at 22° and 30°C. Femoral fracture impaired significantly healing of skin incisions and collagen formation in s.c. polyvinyl alcohol sponges when rats were kept at 22° postoperatively, but this difference was much less for rats at 30 °C. Wounded rats with fractures ate less after injury when offered food ad libitum particularly those kept at 30 °C. Despite this, weight losses were similar for rats at 22° and 30°. When the rats of all groups at 22° and 30° (with and without fracture) were pair-fed against rats with fracture at 30 °C., weight loss was less for rats at 30 °C and their urinary nitrogen excretion relative to nitrogen intake was less than that by corresponding rats at 22°. At 22°, fractured rats excreted significantly more nitrogen relative to food intake than pair-fed sham controls, but at 30°C., this ratio was similar for rats with and without fracture.

Testosterone propionate, s.c., decreased urinary nitrogen excretion by rats with femoral fracture maintained at 22° or 30° after injury, but had no salutary effect on wound healing in those rats or the pair-fed sham-fractured controls. In fact, testosterone slowed healing of the rats with fracture, particularly as judged by the collagen content of the sponge granulomas.

E. Effects of Supplemental Vitamin A

(1) Background

Serious injury causes metabolic and nutritional changes, impaired healing (established in experimental animals), and decreases in host defense mechanisms leading, all too frequently, to increased morbidity and mortality. These factors are all closely interrelated and it is our overall hypothesis that supplemental vitamin A and β carotene modulate each of them in ways which lessen their impact on one another and mitigate the resultant physiological and clinical consequences.



We have been investigating various aspects of the metabolic reaction to injury, wound healing, infection, and vitamin A for a number of years(1-6). We began these experiments based on our hypothesis that serious trauma increases the "requirement" for vitamin A and as pointed out in previous Progress Reports our studies with experimental animals have indicated that there is, in fact, an increased requirement for vitamin A after injury under conditions where pre-existing vitamin A deficiency does not exist. However, only the most minimal data are available regarding the "requirement" of vit. A for seriously injured patients. We have put the word requirement in quotes because what criteria should be used for assessing the requirement are not established - the very few papers dealing with injured or ill patients have dealt largely with measurements of serum vitamin A levels, an inadequate approach because, for example, serum vitamin A levels may be in the normal range for considerable periods of time while the individuals are becoming progressively depleted of vitamin A. Because of lack of information, there is considerable variation in the amounts of vitamin A given to seriously injured patients. The A.M.A. Nutrition Advisory Group Guidelines for Multivitamin Preparations for Parenteral Use (7) adopted in 1975 (and not revised since) recommended 3,300 I.U. vitamin A "... as a daily maintenance dosage for patients (adults, SML) receiving total parenteral nutrition, and are also useful in other situations where administration by the intravenous route is required". No specific mention is made of patients with serious injury, but many surgeons follow this for their injured patients (8) while others give less (9) and others, including ourselves, give more (10, 11).

The amount of supplemental vitamin A required by the injured animal or patient to moderate ("correct") the metabolic, host defense, and wound healing changes is prevention of the classic signs of vitamin A deficiency, e.g., impaired dark-adaptation and failure of epithelial cell differentiation. In fact, the amounts of vitamin A required by the severely injured may well be in the "pharmacologic" range, a view also expressed recently by Malkovsky, Medawar and their associates (12) regarding the effect of supplemental vitamin A on neoplasia. It should be noted that very large doses of vitamin A (100,000 I.U. per day have been given for as long as a year to patients with cancer with only a few isolated instances of vitamin A toxicity (13). Also, supplemental beta carotene, which in some of our experimental studies of neoplasia, radiation

and stress ulcer and in those of others aling with neoplasia has effects similar to those of supplemental vitamin A, has minimal toxicity. It is important to determine whether supplemental beta carotene will act similarly to supplemental vitamin A in regard to moderating the metabolic response to injury, the altered host defense mechanisms and the impaired wound healing of injured animals.

Metabolic Changes After Injury; Effect of Supplemental Vitamin A

Information regarding possible alterations in vitamin A metabolism after injury and its role in the metabolic reaction to injury is limited. What data are available suggest that there may be an increased requirement for vitamin A after injury. Decreases in serum vitamin A(32,33,34)and retinol binding protein and prealbumin have been reported following burns and elective surgery(35). In the latter patients, urinary excretion of vitamin A and retinol binding protein increased. Serum vitamin A fell in psychiatric patients during short-term hyperpyrexia induced by external heat(36). Liver vitamin A was low at autopsy in patients dying following burns or other injuries(33,37). In animals, intermittent daily immobilization of rats led to decreases in serum, liver, testes and kidney vitamin A and an increase in adrenal vitamin A(38). Paravertebral skin incisions of rats ingesting a vitamin A-free diet for two weeks did not result in a change in liver or serum vitamin A (39). Kagan (40) found that rats with subcutaneous abscesses as the result of repeated injections of turpentine and sweet almond oil showed sustained decreases in serum vitamin A concentration and in liver vitamin A concentration and content. Gastrointestinal absorption of vitamin A was not altered, but urinary excretion increased, and the kidney concentration of vitamin A was elevated.

We have found that supplemental vitamin A lessens the weight loss (41) and decreases the urinary excretion of orotic acid by burned rats (42). We interpret the latter as indicating that supplemental vitamin A has modulated the metabolic reaction to injury in ways which reduce the otherwise increased requirement for dietary arginine after injury; orotic aciduria is characteristically seen in dietary arginine deficiency.

Effect of Injury on Wound Healing; Effects of Supplemental Vitamin A

There are little objective, quantitative data of how severe injury affects wound healing in patients. However, the evidence is clear from our studies with experimental animals that wound healing is impaired after serious injury (burns, femoral fracture) (41, 43, 44); this impairment occurs in the absence of hemodynamic, respiratory or renal dysfunction.

Ehrlich, Hunt and their associates (45)(46) have shown that supplemental vitamin A restored towards normal the breaking strength of incisions and the closure of open skin wounds in cortisone treated rats, and Lee and Tong(47) have demonstrated that the retardation of healing by salicylates, hydrocortisone and prednisone can be reversed by applying retinoic acid to the wound.

Clark and Colburn (50) had found that large doses of cortisone result in the rapid depletion of vitamin A from the liver and kidneys of rats and we (51) found a decrease in the vit. A contents of the thymus and adrenals, especially the thymus, and in stress ulcers in mice subjected to stress by the application of a partial body cast. Subsequently, Atukorala, Basu and Dickerson (52) showed also that large doses of corticosterone given to rats led to rapid loss of vitamin A from plasma, liver, adrenals, and especially thymus. These decreases were prevented when supplemental vitamin A was given with the corticosterone.

Some of our studies dealing with some aspect of these matters are described elsewhere (53, 54).

Effect of Injury on Host Defense Mechanisms; Effects of Supplemental Vitamin A

Serious injury leads to impairment of a wide variety of host defense mechanisms, local and systemic (55-61). There are data showing that nutritional vitamin A deficiency increases the incidence of infections (62, 63). There are also some data showing that supplemental vitamin A given to uninjured animals, to animals subjected to immobilization and fasting, or to animals with neoplasms ingesting a normal diet increases various host defense factors (64-66). However, data about how supplemental vitamin A affects the otherwise impaired host resistance following injury are very limited.

To elaborate: A number of investigators have shown in experimental animals and patients that injury (including burns) leads to alterations in key host defense components. These include alterations in complement, e.g., activation and depletion of the alternative complement pathways (67), decreased neutrophil bactericidal activity (68) and serum IgG (69,70), transient decrease in fibronectin (71), inadequate interleukin 2 production, which appears responsible for the depressed response to T-cell mitogens (72), appearance of a serum factor(s) (peptide(s)) which suppresses lymphocyte mitogenic response to PHA and ConA (73); such sera transfused into mice suppressed their immune response and increased the lethality of Listeria monocytogenes infection (74), alteration in T-cell lymphocyte subsets, with a decrease in the ratio of helper cells to suppressor cells (72-81), decreased delayed type hypersensitivity skin reactions (82,83), and decreased humoral immune responsiveness, e.g., to tetanus toxoid (84).

Supplemental vitamin A increases cell-mediated immune responses in experimental animals (85-91). Supplemental vitamin A given to normal mice hastens skin allograft rejection (92) and Medawar, Malkovski and colleagues (93,94) found that vitamin A interferes with induction of neonatal tolerance. Supplemental vitamin A also increases resistance of mice to a variety of experimental neoplasms; the basis of this is immunologic, largely thymic dependent (94,95) and a reflection of alterations in T lymphocyte subsets (94, 96-100).

Vitamin A also plays an important role in humoral defense mechanisms. Several groups of investigators have found a decrease in the number of antibody producing cells and in antibody production following various antigenic and microbial challenges in vitamin A deficient rats (101, 102). In contrast, others have shown that these responses are increased in normally nourished rats given supplemental vitamin A (103-105).

Supplemental vitamin A also increased the ability of alveolar macrophages to phagocytize opsonized sheep red blood cells and increased the in vitro tumoricidal activity of the macrophages (106).

There are very limited data on how supplemental vitamin A affects host resistance following injury. Cohen and colleagues (107) have shown that the severity of Ps. aeruginosa infections in burned animals is lessened when supplemental vitamin A is given. Fusi, Kupper, Green and Ariyan (110) have recently shown that supplemental vitamin A mitigates the depression of the one way mixed lymphocyte reactions in burned mice. Cohen and his colleagues (111) have found that supplemental vitamin A given to patients undergoing elective abdominal operations lessens the ealy postoperative depression in lymphocyte reactivity.

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(2) Our experimental studies

(a) Effects of Marginal Vitamin A Intake on Responses to Minor Wounding

1. Rats on a marginal intake of vitamin A, sufficient to maintain near normal growth, lost weight and became hypoglycemic after relatively minor wounding (dorsal skin incision and polyvinyl alcohol sponge s.c. implants) despite little difference in food intake compared with their wounded normal controls. About half of the rats on marginal vitamin A intake died while none of the wounded controls died.

2. Wound healing was markedly impaired in the A-deficient rats. They formed less reparative collagen and that collagen which was formed was less cross-linked than normal.

3. Administration of vitamin A postoperatively to such rats ameliorated these adverse effects of wounding.

(b) Effects of Vitamin A Supplementation of Normally Nourished Rodents

a. Effects on Wound Healing

Supplemental vitamin A given to healthy rats ingesting a commercial rat chow containing more vitamin A than the NRC recommended allowance for normal rats and which supports normal growth, longevity, reproduction, and lactation of normal rats has a number of beneficial effects on wound healing (dorsal skin incisions, s.c. implanted polyvinyl alcohol sponges):

1. Lessens the weight loss due to anesthesia, operation, and injury (113).

2. Promotes the early inflammatory reaction to wound healing (1-3 days post-operatively). This is important, since subsequent healing is affected to a major degree by the nature of the early inflammatory reaction.

3. Increases the vascularization of reparative tissue (3-7 days post-operatively).

4. Affects the changes which occur after injury in peripheral white blood cells, particularly the polymorphonuclear cells lymphocytes and monocytes. In uninjured rats, supplemental vitamin A induced in three to four days a temporary circulatory leukocytosis characterized principally by lymphocytosis and monocytosis. These changes in the blood picture persisted one day after femoral fracture. On the second and third day post-fracture, the lymphocyte values returned to normal while the monocytosis persisted.

5. Increases the influx of monocytes/macrophages into the wound site adjacent to a femoral fracture. The role of the macrophages in healing is well documented. We had shown earlier that the neutrophils do not play a fundamental role in wound healing, other than to help combat wound infection, a finding confirmed later by Simpson and Ross.

6. Increases reparative collagen in the skin incisions and in s.c. implanted polyvinyl alcohol sponges and in the regions just subjacent to the skin incision and surrounding the s.c. polyvinyl alcohol implants.

7. Wound healing is impaired in injured animals (rats, guinea pigs) while liver regeneration following partial hepatectomy is accelerated. These findings are consistent with ^{15}N glycine metabolic studies we carried out at that time. Supplemental vitamin A ameliorates the otherwise impaired wound healing of injured animals (unilateral or bilateral femoral fractures) by increasing the rate of gain of strength of skin incisions and accumulation of reparative collagen in the s.c. implanted sponges. The impairment in healing was greater in those rats with bilateral femoral fractures than in those with unilateral femoral fractures as judged by the

formation of reparative collagen. The rats with fracture showed an expected significant increase in urinary nitrogen excretion. In some experiments, the rats were fed ad libitum while in others pair-feeding was carried out; the results were similar in both instances.

We found in other experiments that supplemental vitamin A:

8. Increases the incidence and severity of experimental intra-abdominal post-operative adhesions following ligation of a small fold of peritoneum in mice.

9. Increases the hydroxyproline content of both normal colon and the colon anastomotic site, and the bursting strength both of the anastomotic site and of the normal colon segments. The anastomotic breaking strength peak values did not differ significantly from the control, but the area under the breaking strength curve (an expression of the energy required to break the anastomosis) was three times greater in the vitamin A supplemented rats than in the controls. There was a dramatic change in the "elasticity" of the healing anastomotic site tissue. Vitamin A supplementation thus has a beneficial effect on the healing of colon anastomoses in rats.

In other experiments, we found that vitamin A supplementation increases gastrointestinal collagen content in normal rats and prevents the decrease in tissue collagen following injury.

10. Increases the accumulation of reparative collagen at the site of rat aortic anastomosis and adjacent unwounded aorta without affecting collagenase activity at either location, indicating that the effect of supplemental vitamin A is largely on reparative collagen synthesis.

11. Vitamin A added to the culture medium of 3T3 fibroblasts slowed cell multiplication rate and increased the fibroblast layer hydroxyproline content considerably, including during the period of logarithmic growth of the fibroblasts. The data and those of others suggest to us that one way in which supplemental vitamin A affects the wound healing is by accelerating fibroblast differentiation as manifested by morphologic changes, alterations in cell growth rate and collagen accumulation.

12. Prevents the decreased reparative collagen accumulation, the thymic involution (weight and number of thymic lymphocytes), and lessens the body weight loss, adrenal and kidney enlargement, and leukopenia (principally the lymphopenia) of streptozotocin diabetic rats, an effect that is independent of any effect of supplemental vitamin A on the hyperglycemia ($>$350mg%) polydypsia, glycosuria ($>$2%), and polyuria. These experiments were based on our hypothesis from data of Goodson and Hunt dealing with wounds in streptozotocin diabetic rats treated with insulin at varying times post-operatively that there is an impairment in the early inflammatory response after wounding in the diabetic rat and that this would be alleviated by supplemental vitamin A: we had shown earlier that supplemental vitamin A increases the early inflammatory response to wounding.

13. Prevents the weight loss, impaired wound healing, thymic involution (weight and number of thymic lymphocytes), adrenal enlargement, and hemorrhage of Sprague-Dawley rats given cyclophosphamide, without interfering with its anti-tumor action, but, in fact, increasing it. Also, supplemental vitamin A decreases mortality of Fischer rats subjected to wounding and cyclophosphamide.

14. We have carried out several studies of the effects of supplemental vitamin A on radiation injury and combined radiation injury and trauma. We undertook these in part because of our studies with streptozotocin and cyclophosphamide, radio-mimetic agents, and postulated that supplemental vitamin A would be protective. Our experiments showed that supplemental vitamin A:

a. decreases the weight loss, leukopenia (principally the lymphopenia), thymic involution (weight and number of thymic lymphocytes), and adrenal enlargement of rats following x-irradiation of one extremity. This was so whether the supplemental vitamin A was begun the day before or promptly after radiation.

b. Increases the resistance of rats to whole body gamma-irradiation-shifts the dose:response ($LD_{50/30}$) curve to the right, lessens the leukopenia (principally lymphopenia), and weight loss. These ameliorating effects of supplemental vitamin A are

evident event when the supplemental vitamin A is begun 2-3 days after radiation.

c. Prevents the impaired wound healing and lessens the weight loss, leukopenia, thrombocytopenia, thymic involution, adrenal enlargement, decrease in splenic weight, and gastric ulceration of whole-body radiated (750-850 rads) wounded rats. This was so whether the supplemental vitamin A was begun vefore (2 or 4 days) or after (1-2 hours to 4 days) radiation and wounding. The supplemental vitamin A was more effective when started before or up to 2 days after radiation and wounding.

b. Effects on Gastroduodenal Ulceration

We found that supplemental vitamin A prevents or mitigates gastrointestinal ulcerations in a number of other experimental conditions, e.g., it inhibits the ulcerogenic and temperature lowering actions of fasting and restraint of rats and mitigates the ulcerogenic actions of aspirin, and diaminotoluene.

c. Effects on Life-threatening Trauma and Toxicities

Because of our earlier studies showing an increased requirement for vitamin A by rats subjected to moderate injury (femoral fracture), we hypothesized that supplemental vitamin A would likely influence in salutary ways the morbidity and mortality of mice subjected to a severe and life-threatening trauma. We found that supplemental vitamin A:

1. Minimizes the occurrence of gastric and duodenal ulceration and death of rats following Noble-Collip Drum traumatic shock.

2. Abrogates the potentiation by prior whole-body irradiation ($LD_{10/30}$) of the lethality of thermal injury.

3. Abrogates the lethality of aspirin given in an amount lethal for 30% of rats ingesting our standard rat chow.

d. Effects on Metabolic Reaction to Injury: Effects of Injury on Vitamin A Metabolism

1. As mentioned, we found that supplemental vitamin A mitigates the weight loss after wounding. Our previous work showed also that adrenal enlargement and thymic involution occurring during stress was obviated by feeding supplemental vitamin A, an effect we attributed in part to vitamin A's antagonism to glucocorticoids released during stress. Atukorala and his colleagues have shown a similar effect of vitamin A in rats injected with corticosterone. In other experiments, we found that supplemental vitamin A lessens the otherwise marked rise in thymic and liver corticol receptor protein induced in mice subjected to partial body casting.

2. Because increased secretion of catecholamine is a major factor after injury, we studied also the effect of injected epinephrine and norepinephrine on the thymus and adrenal glands. We found that supplemental vitamin A prevented the thymic involution and the adrenal enlargement induced by epineprine and prevented the thymic involution and lessened the adrenal enlargement induced by norepinephrine.

3. In a series of other studies, we had shown that injured rats have an increased requirement for dietary arginine. We postulated that supplemental vitamin A would modulate the metabolic reaction to injury in ways which would prevent the increased dietary arginine requirement. To test this, we measured urinary orotic acid excretion after severe burns of rats. Our reason for this was that increases in urinary orotic acid are well documented in experimental dietary arginine deficiency. If, as we have postulated, there is an increased requirement for exogenous arginine following severe injury and, unless met, a relative deficiency of arginine develops, we predicted that a severe burn would cause orotic aciduria and that supplemental arginine would mitigate this. We further hypothesized that supplemental vitamin A in the absence of supplemental arginine would also mitigate the orotic aciduria.

Rats were the experimental animal. There was an abrupt, marked rise in urinary orotic acid following burning (third degree burns, 30% body surface area) in the rats ingesting a standard chow diet. The increase was evident in the first two days after burn, rose still higher by days 5-6, peaked at days 9-10 and was still very high at days 13-14, which was as long as the measurements were made. In the burned rats ingesting the supplemental arginine there were increases in urinary orotic acid during the first six days, which were not statistically different from the early increases shown by the rats ingesting the standard chow diet. However, thereafter, the level of urinary orotic acid fell sharply in the arginine supplemented rats, in marked contrast to the rats ingesting the standard chow. The rats supplemented with vitamin A behaved similarly to those supplemented with arginine. There was no greater effect when both the supplemental vitamin A and arginine were given. These data support our view of the modulating effect of supplemental vitamin A on the metabolic alterations which follow serious injury, one major consequence of which is an increased requirement for arginine.

e. Effects on Host Defense Mechanisms

A series of studies showed that supplemental vitamin A has important effects on the thymus and immune responses. We have found that supplemental vitamin A:

1. Increases thymic size and number of thymic lymphocytes in uninjured intact rats and mice, i.e., supplemental vitamin A has a thymotropic effect. This thymotropic effect is independent of the adrenals, since it is seen in adrenalectomized rats.

2. Accelerates skin allograft rejection.

3. Injury is immunosuppressive. Supplemental vitamin A lessens substantially the abrupt decrease in the size of the thymus and the number of thymic lymphocytes and the increase in adrenal size which characteristically occur following injury.

4. Moderate stress (wearing of a partial body cast) is thymolytic and immunosuppressive, e.g., rejection of skin allografts is delayed. Supplemental vitamin A inhibits these reactions as well as the characteristic weight loss and increased adrenal size.

5. As mentioned, prevents the thymic involution and adrenal enlargement which follows the injection of epinephrine and prevetns the thymic involution and lessens the adrenal enlargement which follows the injection of norepinephrine.

6. As mentioned, mitigates the thymic involution (decrease in thymic weight and number of thymic lymphocytes) and adrenal enlargement following local (one extremity) and whole-body gamma-radiation.

7. Prevents the adrenal enlargement and hemorrhage and thymolytic effects of toxic chemicals such as dimethylbenz( )anthracene.

8. Prevents the decrease in thymic lymphocytes, thymic size, and peripheral blood lymphocytes and the increase in adrenal weight in rodents subjected to experimental tumors.

9. Increases the resistance of rodents (both uninjured and injured) to various experimental tumors (e.g., Moloney Sarcoma Virus, C3HBA, DMBA). This effect is independent of the adrenals since it is seen in adrenalectomized animals.

10. One of us (E.S.) in collaboration with Levine and Pruitt has found that supplemental vitamin A decreases the mortality of burned mice subjected to virus (Moloney Sarcoma) infection.

11. Supplemental vitamin A increases the resistance of burned rats (3^{o} burn, 35% body surface) to the purposeful local contamination of the burns with a very highly virulent strain of Ps. aeruginosa.

12. Because previous work in our laboratory showed that dietary supplementation with vitamin A enhances certain immune responses of normal and injured rats and increases peritoneal adhesion formation in mice, we postulated that vitamin A would

likely help control intra-abdominal sepsis. We found that vitamin A dietary supplementation does have a significant protective effect in the rats whose ligated cecum was perforated with an 18-gauze needle. This was manifested by increased survival prevention of postoperative hypothermia, maintenance of peripheral WBC counts at normal or above-normal values, and better localization of the intra-abdominal inflammatory process. Dietary supplementation with beta carotene had a lesser protective effect.

(c) Studies Not Reported in Previous Progress Reports

The level of supplemental dietary vitamin A we have used (150,000 I.U. vitamin A/kg diet) is not toxic for rats and mice; we have maintained mice on this supplement for 2 years without evidence of toxicity. The level of vitamin A supplementation we have used is similar to those used by Hunt and his colleagues in their wound healing studies, by several groups in their stress ulcer studies, and by Cohen and associates for increasing resistance to infection. The level of vitamin A to be used is less, than those used by several groups of workers for the treatment of prevention of hypocarbon-induced tumors and much less (on a weight corrected basis) than are used in the treatment of patients with malignancies, for stress ulcer prevention or prevention of surgically induced immune depressions.

a. Effect of Supplemental Vitamin A on the Healing of Rat Colon Anastomoses

We reported previously that supplemental dietary vitamin A increases the hydroxyproline content of both normal colon and the colon anastomotic site, and the bursting strength both of the anastomotic site and of the normal colon segments. The anastomotic breaking strength peak values did not differ significantly from the control, but the area under the breaking strength curve (an expression of the energy required to break the anastomosis) was three times greater in the vitamin A supplemented rats than in the controls. There was a dramatic change in the "elasticity" of the healing anastomotic site tissue. Vitamin A supplementation thus has a beneficial effect on the healing of colon anastomoses in rats.

In other experiments, we found that vitamin A supplementation increases gastrointestinal collagen content in normal rats and prevents the decrease in tissue collagen following injury. We have found recently in similar experiments that there was no difference in collagenase activity at the healing anastomotic site in the vitamin A supplemented and control rats. The data suggest then that the beneficial effect of supplemental vitamin A on the healing of rat colon anastomoses is a reflection of increased collagen synthesis.

b. Effect of Supplemental Vitamin A on Colon Anastomosis Healing in Rats Exposed to Preoperative Radiation

These studies are a follow up of our earlier investigations which showed that supplemental vitamin A accelerates healing of colon anastomoses in otherwise normal rats and counteracts the impaired healing of skin incisions in rats exposed to whole body x-radiation.

The increased risk of impaired healing and anastomotic breakdown of bowel anastomoses after radiation are well known major complications. In view of our just mentioned earlier studies, we postulated that vitamin A supplementation would have a beneficial effect on colon anastomosis healing following preoperative radiation.

Male Sprague-Dawley rats (n=120) weighing approximately 250-300 g were placed in individual steel two-mesh wire cages and acclimatized to our laboratory conditions (temperature 26±1°C, relative humidity 40% and a 14 hour day/10 hour night cycle) for 1 week prior to the beginning of the experiment. Rats were then divided randomly into 4 groups of 30 rats each. Groups 1 and 3 were fed a standard commercial rat chow containing 15 IU vit. A and 6.5 ppm beta carotene per gram of diet, which is several times the National Research Council's Recommended Daily Allowance of vitamin A for normal rats. This chow supports normal growth, fertility, pregancy, lactation, and longevity of normal rats. Groups 2 and 5 ate the same chow supplemented with 150 IU of retinyl palmitate per gram of diet. All rats ate and drank tap water ad libitum.

Rats were started on their respective diets 7 days prior to irradiation and maintained on the same diets throughout the experiment. Two Gy x-radiation were delivered to the abdomen and pelvis of rats (i.p. pentobarbital anesthesia) in Groups 3 and 4; lead shields defined the radiation field and protected the rest of the body.

All rats (both radiated and non-radiated) underwent colon anastomosis seven days later using i.p. pentobarbital anesthesia. The distal colon was divided 1½ inches proximal to the anus, and a one layer anstomosis was performed using interrupted 4-0 silk sutures. On the 7th postoperative day, all animals were killed with ether overdose and the entire colon was excised. Bursting strength measurements were made at the anastomotic site and a segment of adjacent proximal colon. Collagen content was estimated by measuring the hydroxyproline content at both the anastomotic site and the adjacent proximal colon.

Food and water intake was comparable in the vitamin A supplemented and control chow groups as were the patterns of weight change.

Radiation resulted in reduced hydroxyproline content (Table 1) and bursting strength (Table 2) at the anastomosis and the proximal normal colon in both control and vitamin A supplemented animals. Vitamin A supplementation, however, significantly ($p < 0.02$) mitigated the radiation effect. The hydroxyproline content was higher at the anastomosis in both radiated and non-radiated animals following vitamin A supplementation (Table 1). In the proximal normal colon segment, although the hydroxyproline content was higher in vitamin A supplemented animals, with and without radiation, the increase was not statistically significant (Table 1). Colon bursting strength was significantly greater in the vitamin A supplemented rats both at the anastomosis and at the proximal normal colon segment, in both radiated and non-radiated groups (Table 2).

We have shown in these and other studies that supplemental vitamin A has both local and systemic effects which result in improved healing of colonic anastomoses in normal and irradiated animals, and enhanced resistance of animals to radiation. Our findings suggest that dietary supplementation with vitamin A may play an important role in the clinical setting.

Table 1

Effect of supplemental Vitamin A on the Collagen Content of Normal and Anastomotic Colon Segments with and without Preoperative Irradiation

	Hydroxyproline (mg/g tissue ± S.E.M.)	
	Anastomotic Segment	Normal Colon Segment
Groups		
1. Control diet, no irradiation	1.90±0.08	3.50±0.26
2. Vitamin A diet, no irradiation	2.50±0.18	3.70±0.30
3. Control diet, irradiation	1.10±0.06	2.80±0.18
4. Vitamin A diet, irradiation	1.90±0.11	3.20±0.30
P values		
Group 1 vs. 2	<0.02	NS
Group 3 vs. 4	<0.02	NS
Group 1 vs. 3	<0.02	<0.02
Group 2 vs. 4	NS	NS

Table 2

Effect of supplemental Vitamin A on the Collagen Content of Normal and Anastomotic Colon Segments with and without Preoperative Irradiation

	Bursting Strength (mm Hg ± S.E.M.)	
	Anastomotic Segment	Normal Colon Segment
Groups		
1. Control diet, no irradiation	160±9.0	205±13.0
2. Vitamin A diet, no irradiation	200±13.2	226±14.3
3. Control diet, irradiation	102± 4.1	129± 7.0
4. Vitamin A diet, irradiation	120± 6.0	190±11.7
P values		
Group 1 vs. 2	< 0.02	NS
Group 3 vs. 4	< 0.02	< 0.02
Group 1 vs. 3	< 0.02	< 0.02
Group 2 vs. 4	< 0.02	NS

c. Effect of Supplemental Vitamin A on the Healing of Rat Aorta Anastomoses

In our last Progress Report, we reported that supplemental dietary vitamin A increases the accumulation of reparative collagen at the site of rat aortic anastomosis and adjacent unwounded aorta without affecting collagenase activity at either location, indicating that the effect of supplemental vitamin A is largely on reparative collagen synthesis (126). We have recently found in similar experiments that dietary vitamin A supplementation leads to a significant increase in the rate of gain of aortic anastomoses bursting strength (Table 3).

d. Vitamin A: Induced Fibroblast Differentiation in vitro

We have reported in our 1 November 1983 Progress Report that vitamin A added to Balb 3T3 mouse fibroblasts in tissue culture resulted in induction of cell differentiation as manifested by a decrease in all growth rate, enhanced collagen accumulation, and morphologic changes. We now report our findings of experiments to determine whether the vitamin A affected collagenase activity of the cultured cells. For this, Balb 3T3 mouse fibroblasts were incubated with control medium or media containing vitamin A or retinoic acid at various concentrations. At different time intervals, cells were harvested with trypsin (0.25%) and washed three times with phosphate-buffered saline solution, and a suspension of 4×10^6 cells per ml was obtained. Two tenths of one milliliter was then placed in the center of collagen plates. The circumference of the area lysed was marked on the underside of the plate daily, traced onto paper. and then measured by planimetry. Each assay was done in quadruplicate. Fig. 3 shows that neither added vitamin A nor retinoic acid affected the collagenase activity of the cell layer.

Increased reparative collagen and enhanced fibroplasia due to supplemental vitamin A has been demonstrated in several laboratories with various in vivo animal models. The mechanism of this effect, however, is not known. The enhanced collagen accumulation may be due to increased collagen synthesis, decreased collagenolysis, or both. Using an in vitro fibroblast culture system, we demonstrated that vitamin A or retinoic acid induces a decrease in cell growth rate and an increase in cell layer collagen content. At the same time, no change in collagenase activity was noted after vitamin A or retinoic acid cell culture treatment. Using rheumatoid synovial cells, Brinkerhoff et al. (1) were able to show that vitamin A inhibits collagenase production. We could not demonstrate such an effect, however, using a system of normal fibroblasts. Our results thus strongly suggest that vitamin A and retinoic acid enhance collagen synthesis, which in turn results in increased collagen accumulation. The decreased cell growth rate and enhanced collagen synthesis suggest that vitamin A and retinoic acid induce cell differentiation in this in vitro fibroblast culture system. This is supported by the morphologic changes seen in light microscopic examination, which show cells with loss of rounded shape and assumption of a spindle cell form with "dendritic projections. In other experiments, Patt et al. (1) showed that retinol induces a density-dependent growth inhibition of 3T3 fibroblasts; associated changes in cell surface membrane [illegible] level, ganglioside contact response, and large, external, transformation-sensitive protein were also observed. These results suggest that cell surface changes result in contact inhibition of cell growth. However, in the culture system and under the conditions of our study, we observed changes early during the logarithmic phase of growth when cells were not contact inhibited and not just when cells reached confluency and a plateau after vitamin A and retinoic acid addition. This suggests that these agents do not just enhance contact inhibition but rather induce fibroblast differentiation.

In summary, supplemental vitamin A has a beneficial effect on wound healing in vivo by enhancing fibroplasia and collagen accumulation. Data obtained with an in vitro system of cultured fibroblasts strongly suggest that this beneficial effect is due to induction of fibroblast differentiation manifested by increased collagen synthesis, decreased growth rate, and morphologic changes.

Table 3

Bursting Strength of Abdominal Aortic Anastomosis

Experimental Group	Number of Rats	Bursting Strength* (mm Hg)
Control Group	7	251.0 ± 17**
Vitamin A Supplemented Group	7	3021 ± 19

* Results are expressed as Mean ± S.E.M.

** $P < 0.02$

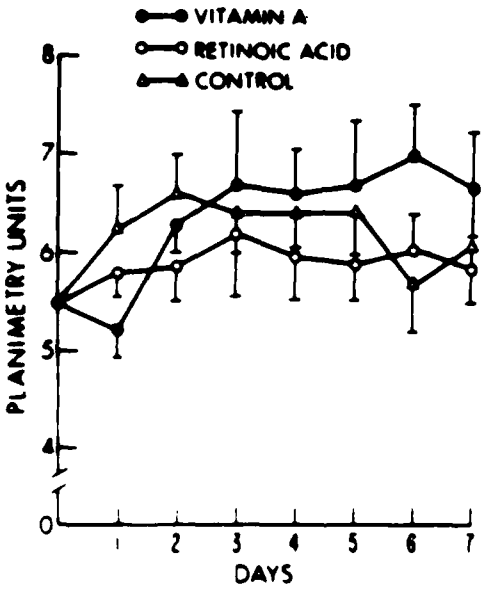

Fig. 1. The effect of vitamin A and RA on collagenase activity. Results are expressed as planimetric units ± SEM and represent mean values from five plates.

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e. Effect of Supplemental Vitamin A on Tissue Vitamin A Contents of Stressed Rats

The vitamin A content of tissues of control and stressed rats were determined in rats receiving our standard chow diet. The stress consisted of a partial body cast applied to the thorax and kept there for three days. Casting lowered the vitamin A content of tissues of the rats ingesting the control chow: thymus, 99%, liver, 7%, adrenals, 18%, perirenal fat (white), 0%, plasma, 17%. Thus, among the organs examined, the thymus was most affected by casting stress in terms of vitamin A loss.

This supports our other experiments and view that an important role of vitamin A is to support thymus-mediated immune reactions. In other experiments, using purified diets, serum vitamin A levels dropped promptly following third degree burns of rats (35% body surface area).

f. Prevention of Cysteamine.HCl-induced Duodenal Ulcer Formation in the Rat by Dietary Vitamin A Supplementation

Peptic ulcer disease remains a major source of morbidity and mortality in clinical practice. The precise etiology and pathogenesis of peptic ulcers are not known, but a majority of investigators agree that duodenal ulcers are primarily due to acid hypersecretion. Work in our laboratory has demonstrated that supplemental dietary vitamin A enhances the collagen content of the intestine of normal and injured rats, increases the early inflammatory response to wounding, enhances the healing of colon anastomoses (1) and accelerates healing of skin incisions (2). Vitamin A has been shown to increase the production of mucus by the stomach and duodenum (3,4). We and Chernov (5) have shown that dietary supplementation with vitamin A decreases the incidence of stress and aspirin induced ulcers in the stomach. In view of these various findings, we carried out experiments to test our hypothesis that vitamin A would prevent duodenal ulcer formation in the rat. The experiment model we chose was the intragastric administration of cysteamine.HCl. A major way cysteamine-HCl induces duodenal ulcer formation is by enhancing gastric acid secretion. Adult male rats were divided into two groups: (1) rats fed a standard rat chow (Purina) (15 IU vitamin A/g diet) containing two to three times the National Research Council Recommended Daily Allowance for vitamin A for normal rats: (2) rats fed the chow supplemented with 150 IU of vitamin A palmitate per/g chow. One week later, all rats were given 1 ml of cysteamine-HCl (135 mg) intragastrically. The rats were maintained on their respective diets. Two days later, all rats were killed with ether, the stomach and duodenum excised, and examined for the presence of ulcers (Table 4). No gastric ulcers were found in either group. There was a statistically significant decrease in the incidence of duodenal ulcers in the vitamin A-supplemented group when compared to the control group ($p < 0.01$) 48 hrs following cystemina-HCl administration; 32% of the vitamin A-supplemented rats developed a duodenal ulcer whereas 74% of rats fed standard chow had duodenal ulcers. Most rats had a single duodenal ulcer in the first part of the duodenum, occasionally a second ulcer was noted in the same area. Dietary supplementation with vitamin A had no effect on the increased gastric acid production induced by the cysteamine.HCl (Table 5). Vitamin A administration immedately after cysteamine.HCl intragastric instillation did not affect the incidence of duodenal ulcer formation (Table 6). Cysteamine.HCl induces duodenal ulcers by increasing gastric acid output(6,7,8,9,10,11) and gastrin production (8,9,10). It also inhibits acid neutralization in the duodenum (12,13) and increases hydrogen ion back diffusion in the duodenal mucosa(13). Cysteamina-HCl has also been shown to decrease somatostatin production (14,10,15) and to inhibit gastric empyting(17). When incubated with human normal and cystinotic fibroblasts, the uptake of L-proline is decreased with significant alterations in the cellular amino acid pools (16). In the present study, we demonstrated an early increase in gastric fluid content and

enhanced total titratable acidity in the stomach following intragastric cysteamine.HCl administration as shown previously by others (6,7,8,9,11).

Dietary supplementation with vitamin A improved animal survival and resulted in a dramatic reduction in both the incidence of duodenal ulcers and the number of duodenal ulcers per animal. Dietary supplementation with vitamin A had no effect on gastric total titratable acidity. It thus seems that the beneficial effect of vitamin A in this system is not mediated through a decrease in gastric acid output. We have shown previously that dietary supplementation with vitamin A results in increased collagen content in the gastrointestinal tract(1) and improved bursting strength of colon anastomoses in the rat(1). Vitamin A has been shown to improve wound healing by stimulating the early inflammatory response (2) and increasing fibroplasia and collagen accumulation (18). It has also been shown to antagonize the inhibitory effect of cortisone on the healing of skin wounds in rats (19) and to enhance the formation of peritoneal adhesions in mice (20). De Luca et al. (21) found that protein synthesis by the rat small intestinal mucosa is depressed at the translational level in vitamin A deficiency. Vitamin A dietary supplementation may also prevent duodenal ulcer formation by increasing mucosal resistance to injury by other mechanism(s) possibly by increasing mucus production (21, 3, 4).

Improved animal survival in the vitamin A-treated group could be due to the decrease in the incidence and severity of duodenal ulcers in these rats and/or due to improved host resistance to injury (2,22). It has previously been shown in our laboratory that vitamin A dietary supplementation has a thymotropic effect in rats and mice (22), enhances resistance to sepsis in rats undergoing cecal ligation and perforation (24) and enhances resistance to injury (23) in the rat.

Intragastric administration of one dose of vitamin A immediately after cysteamine.HCl administration followed by vitamin A dietary supplementation did not affect the incidence of duodenal ulcer. It appears that pretreatment with supplemental vitamin A is needed in order to alter or decrease the incidence and severity of duodenal ulcers in the rat.

In conclusion, dietary supplementation with non-toxic amounts of vitamin A significantly decreases the incidence and severity of duodenal ulcer formation in the rat. This effect does not appear to be due to an effect of vitamin A on gastric acid production. Our experimental results may have clinical relevance. The cysteamine.HCl-induced ulcers in the rat are true peptic ulcers with a similar distribution (proximal duodenum) association with gastric acid hypersecretion, and block of gastric acid hypersecretion by H_2-blockers. Thus the beneficial effect of vitamin A in this esperimental model suggests that vitamin A dietary supplementation may have clinical usefulness in the prevention and treatment of peptic ulcer disease in man.

(continued next page)

TABLE 4

Incidence of DUs in rats prefed with Vit. A

Dietary supplementation	No. rats with ulcer/total no. rats	% rats with ulcers	No. rats with multiple ulcers[a]
None	20/27	74	4/27
Vitamin A	8/25	32	1/25

[a] $p < 0.01$.
[b] $p < 0.05$.

TABLE 5

Effect of dietary supplementation with Vit. A on gastric acid production

Experimental group	Gastric content volume	Gastric content pH	Total titratable acidity at 4 hr
	ml		*mEq*
A. Control diet	4.19 ± 0.8[a]	1.85 ± 0.4	0.36 ± 0.02
B. Control diet cysteamine-HCl	10.12 ± 0.9	1.93 ± 0.4	0.57 ± 0.03
C. VA-diet	2.99 ± 0.4	1.55 ± 0.5	0.36 ± 0.02
D. VA-diet cysteamine-HCl	8.25 ± 0.8	2.91 ± 0.6	0.50 ± 0.02
	p volumes		
Groups			
A *vs* B	<0.01	NS	<0.05
C *vs* D	<0.01	NS	<0.05
A *vs* C	NS	NS	NS
B *vs* D	NS	NS	NS

[a] Values represent mean ± SEM.

TABLE 6

Incidence of DU in rats given Vit. A intragastrically immediately after cysteamine-HCl intragastric instillation

Dietary supplementation	No. rats with ulcer/ total no. of rats[a]	% of rats with ulcers
None	5/10	50
Vitamin A	5/10	50

[a] p = NS.

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g. Morbidity and Mortality Reduction by Supplemental Vitamin A, Beta Carotene in Mice Given Total-Body Gamma-Radiation

Supplemental vitamin A reduces morbidity and mortality of rats and mice due to the chemotherapeutic agents streptozotocin and cyclophosphamide (1-3). These compounds are described as radiomimetic because, like some forms of ionizing radiation, they give rise to cation or cationoid species that alkylate cellular constituents. Animal responses to radiomimetic compounds are similar to those caused by radiation; therefore, several years ago we studied the effect of supplemental vitamin A on radiation sickness in C3H mice given local non-lethal x-radiation (3,000 rad) in the hindlimb and observed that supplemental vitamin A, begun prior to or directly after irradiation, decreased radiation-induced toxicity, i.e., weight loss, lymphopenia, thymus involution, and adrenal gland enlargement (4). Similarly, supplemental vitamin A or beta carotene, dietary precursor of vitamin A, each diminished systemic toxicity due to local x-irradiation (3,000 rad) in tumor-bearing mice without diminishing the anti-tumor effect of the radiation (5, 6). In fact, in tumor-bearing mice that received both 3,000 rad local tumor radiation and supplemental vitamin A or beta carotene, the antitumor action of local tumor radiation was markedly enhanced. We have reported also previously that supplemental dietary vitamin A reduces significantly the morbidity and mortality of mice exposed to whole-body gamma-irradiation. We now report similar mitigating effects when mice are fed supplemental beta carotene. In view of the above findings, in the present studies, we examined the influence of supplemental beta carotene on the morbidity and mortality of male CBA mice subjected to graded dosages of total-body gamma-irradiation.

The mice (CBA/J, male, 6 weeks old) were housed in a room with a 12-hour day-night cycle, a temperature of 26°C, and relative humidity of about 35%. The basal control ration was a standard powdered commercial laboratory chow which contained 15,000 IU vitamin A/kg diet and 6.4 mg beta carotene (equivalent to 3,000IU vitamin A); therefore, the basal control chow contained several times the National Research Council's Recommended Daily Allowance of vitamin A. The mice were maintained on this diet for 7 days before initiation of the tests.

Experimental diets were prepared every other day from the basal control ration by admixing an additional 90 mg beta carotene to each kilogram of chow. All diets were kept in black plastic bags to prevent photooxidation of the carotenoids. We have used these non-toxic levels of supplementation previously in a number of studies including studies of local x-irradiation (1-6) and have maintained mice in good health on this type of diet for well over a year. All mice drank tap water ad libitum throughout the experiments.

Irradiation conditions - Groups of 30 mice received total-body gamma radiation in a ^{137}cesium small animal irradiator (Gamma-cell-40; Atomic Energy Ltd., Ottawa, Canada). This unit contains two ^{137}Cs sources of approximately 1,800 Ci; one source was positioned above and the other below the exposure cavity to produce a uniformity of ±5% for the 0.66-MeV gamma-radiation within the 30-cm diameter and the 10-cm-high sample chamber. The dose rate within the chamber, calibrated by thermoluminescent dosimeters, was 1.37 Gy/minute. Thus total-body gamma-radiation doses of 4.5-7.5 Gy were delivered in 3.3-5.5 minutes. Forced ventilation was supplied to the exposure cavity during irradiation to remove vapor-phase ions or radicals generated by the radiation.

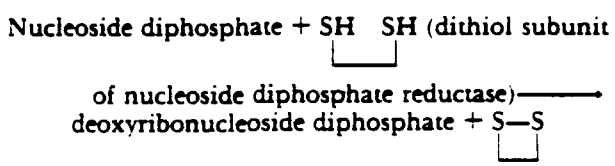
Immediate supplementation post-irradiation - Supplemental beta carotene altered the $LD_{50/30}$; the $LD_{50/30}$ was 510 rad for controls and 645 rad for the beta carotene-supplemented mice. A dose that killed 50% or more of the untreated mice killed none of the mice supplemented with beta carotene (Fig.2).

Supplemental beta carotene mice that died lived longer than did the unsupplemented mice that died and had received the same dose of radiation. Mean survival times of mice were inversely related to the radiation dose and markedly increased at all radiation doses by supplemental beta carotene; the $t_{\frac{1}{2}}$ was also inversely related to radiation dosage (Fig.3).

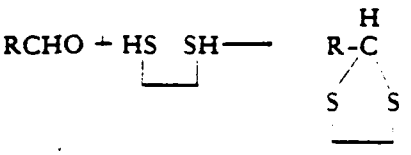
Thymus weights were higher and adrenal gland weights were lower in irradiated mice receiving supplemental beta carotene compared to weights in control mice.

It is not known how supplemental beta carotene protects against total-body gamma radiation death. Carotenoids, particularly beta carotene, reduce not only the direct untoward effects of ultra violet absorption (7) but also its indirect effect, i.e., the cocarcinogenic action of ultra violet radiation in appropriate test animals(8-10). Similarly, some retinoids can inhibit radiation-induced transformation of animal cells in vitro (11). These data may be relevant to our findings because in some instances inhibition of transformation and cocarcinogenicity is specific for carotenoids with vitamin A-like activity, which may be applied after radiation to prevent late changes in carcinogenesis. In other instances, to be active, the carotenoids must be present before UV irradiation; i.e., the carotenoid performs a screening function (8) and interferes wtih absorption of UV rays particularly by nucleic acids. It is doubtful that vitamin A or beta carotene acts as a screen for the energy-rich photons of

x-rays or gamma-rays that produce direct ionization; similarly, the carotenoids would not screen against neutrons that produce ions and radiation, suggesting that the main radioprotective action of beta carotene is due to factors other than screening. However, beta carotene and vitamin A may protect against (or reverse) radiation damage by promoting cell differentiation, as opposed to cell division. Actively dividing cells are more radiosensitive than are nondividing or post-mitotic cells. We speculate that one way vitamin A promotes cell differentiation (and decreases radiation toxicity) is via an interaction of retinal and ribonucleoside diphosphate reductase, an enzyme that regulates DNA synthesis:

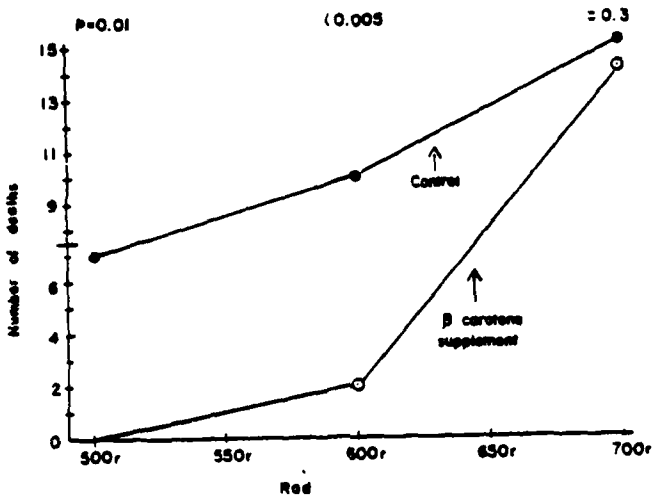


In the presence of excess retinal (RCHO), the following reaction would occur readily:

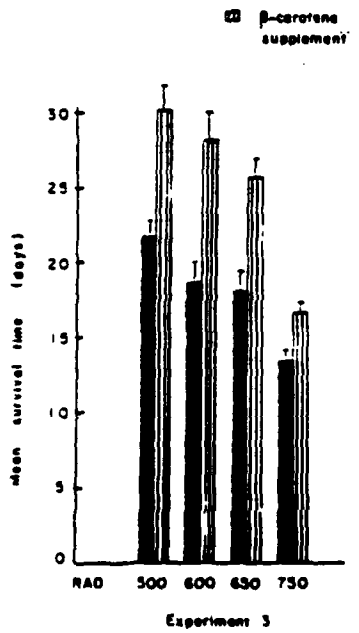


The reaction is likely to have two important physiologic effects: a) conversion of the radiosensitive forms of the enzyme (the dithiol and disulfide) to a more radioresistant thioacetal and b) regulation of DNA synthesis by inhibition of the biosynthesis of DNA precursor nucleotides from ribonucleotides. In the second reaction, vitamin A would favor RNA synthesis and cell differentiation rather than DNA synthesis and cell proliferation. The hypothesis predicts that retinoic acid is less active than retinyl esters because the former is not convertible to retinal. Investigations to test this hypothesis are in progress. This hypothesis was advanced by Drs. Eli Seifter and Jacques Padawer.

The enhanced short-term survival due to supplemental vitamin A and beta carotene may be associated with a special antioxidant effect of these compounds. They may function not so much by trapping free radicals produced directly by radiation, but rather by trapping second-generation or indirectly produced free radicals. For example, free radicals, i.e., compounds with unpaired electrons and therefore of high chemical reactivity, may normally be generated by inner mitochondrial membrane enzymes of the electron transport chain. However, because the transport chain is tighly linked, radicals are not entirely free inasmuch as electrons flow toward the cytochrome system and then to oxygen. If the membrane is damaged (radiation damage, radiation-derived peroxide damage of unsaturated membrane lipids), the flow of electrons from substrates to oxygen may produce discrete free radicals, including superoxide that may then further damage the mitochondrial membrane. Beta carotene and vitamin A possibly act to trap or quench the free radicals and thereby inhibit damage to cells and their membranes. In this way, beta carotene and vitamin A could interrupt the chain of free radical ractions initially caused by radiation damage. Additional discussion of how vitamin A and beta carotene may protect against radiation injury is included in our publications.



TEXT FIGURE 2.—No. of deaths occurring within 30 days of TBR in expt 3. Mice were placed on experimental diet immediately after irradiation. *P*-values were calculated from chi squares.



TEXT FIGURE 3.—Mean survival time = sum of days survived by each animal/15 (No. of animals/group). Diet supplements were started immediately after irradiation. *Bar graphs* are means ± SEM.

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Reduction of Mortality by Supplemental Vitamin A, Beta Carotene in Mice Exposed to Whole-body Gamma-Radiation

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h. Whole-body Irradiation-induced Leukopenia and Thrombocytopenia: Protection by Supplemental Beta Carotene

In mice receiving whole body radiation, supplemental vitamin A reduced death rates, leukopenia, and thrombocytopenia: supplemental beta carotene reduced death rates. We have now studied the influence of supplemental beta carotene on radiation-induced leukopenia and thrombocytopenia. Pairs of male, C57 Bl/6 mice were established. Three days later, white cell and platelet counts were made and the mice received 6 Gy whole body x-irradiation. Cell counts were made at 3-4 days intervals.

Day	0	+1	+3	+7	+10	+14
	Platelets (in thousands/mm^3)					
Control (1)	540	460	420	130	40	125
BC (1)	552	542	555	428	358	347
Control (2)	500	425	425	180	75	175
BC (2)	453	382	298	305	286	235
	White Cells (in hundreds/mm^3)					
Control (1)	67	25	12	25	35	50
BC (1)	62	34	43	68	66	65
Control (2)	75	32	15	32	29	33
BC (2)	65	36	54	75	76	110

Supplemental beta carotene diets contained 90 mg beta carotene/kg. BC moderated the radiation-induced thrombocytopenia and the leukopenia by, we think, preventing destruction as well as stimulating recovery of platelets and leukocytes.

The data are relevant for radiation or radiomimetic chemotherapy.

i. Supplemental Vitamin A Reduced Cyclophosphamide Toxicity in Wounded Fischer Rats

Previous work in our laboratory has shown that vitamin A alleviates the weight loss and inhibited-wound healing in Sprague-Dawley rats treated with cyclophosphamide. Although similarly-treated Fischer rats were more severely affected, vitamin A was equally protective. This study examined the mechanism of cyclophosphamide toxicity in wounded rats and the protective action of vitamin A. Six groups of male Fischer rats were used; three remained unwounded and three received 7.0 cm dorsal skin incisions (pentobarbitol) which were closed with 7 equidistant sutures. Cyclophosphamide-treated rats received 24 mg cyclophosphamide/kg on 4 consecutive days (beginning immediately after wounding). Vitamin A-supplemented groups received 150 IU vitamin A/g diet. Ten days later, WBC and platelets were counted (/mm^3) and wound breaking strengths (WBS) measured (g).

Group	Treatment	WBC	Platelets	Deaths	WBS
1	Control	15,450±2,950	285,000±30,500	0/6	-----
2	CY	3,725±1,575	72,500±14,500	2/6	-----
3	CY+VA	7,575±475	205,500±9,500	0/6	-----
4	Wound (WD)	13,650±300	185,500±10,500	0/6	550±26
5	WD+CY	3,375±1,325	48,500±13,500	6/8	237±24
6	WD+CY+VA	10,125±875	219,500±13,500	0/8	591±27

Cyclophosphamide reduced WBC and platelet counts and wound breaking strengths. It caused 2/6 deaths in the unwounded group and 6/8 in the wounded group. Vitamin A moderated these effects. We conclude that cyclophosphamide inhibition of platelet regeneration results in decreased platelet-derived growth factors that favor wound healing. Because wounding consumes platelets, the combination of wounding and cyclophosphamide is especially toxic. Vitamin A stimulates platelet regeneration and therefore moderates the toxic effects of cyclophosphamide.

F. Effects of Supplemental Dietary Arginine and Ornithine

In our previous Progress Reports we have reported on a number of our studies which have shown that supplemental dietary arginine has wound healing accelerating, thymotropic, and immunogenic effects.

We hypothesized that injury would likely increase the dietary arginine requirement. This stemmed from the observations that (1) the ratio of dietary essential:non-essential amino acids required by the severely injured, previously healthy male approaches that of the child; arginine is a dietary essential amino acid for the growing rat and child but not for the healthy adult rat and human; and (2) arginine is a precursor of proline, an amino acid that is critical for the synthesis of collagen, a component so essential for wound healing.

Our interest in arginine began during the Korean War. when we studied the behavior of individual amino acids in burned rats (Rosen, H. Levenson, S. M., Proc. Soc. Exper. Biol. Med. 83: 91, 1953), and humans (Levenson, S. M., Howard J. M., Rosen, H., Surg. Forum 5: 483, 1954). and in soldiers wounded in battle (Levenson S M., Howard, J. M., Rosen, H., Surg. Gynecol. & Obstet. 101: 35. 1955), including some with renal failure (Rosen, H., Meroney, W. H., Levenson, S. M., Clin. Sci. 17: 653, 1958), and some with hemorrhagic fever (Levenson, S. M., Markelz, R. A., Mason, J., Rosen, H., Commission on Hemorrhagic Fever, 1955), and patients with liver disease (Iber, F. L., Rosen, H., Levenson, S. M., Chalmers, T.C., Lab. Clin. Med. 50: 417, 1957). Since then there have been an increasing number of studies of amino acid metabolism after injury, sepsis, and hepatic and renal failure. While no one has pointed out the unique derangement of arginine metabolism per se, plasma arginine concentrations were often found to be low after injury.

(continued next page)

a. Some Effects of Supplemental Dietary Arginine

1. In an initial series of experiments, using chemically defined amino acid diets with varying levels of arginine, it was found to be a major limiting amino acid for wounded (skin incision; subcutaneous implantation of polyvinyl alcohol sponges) rats but not uninjured rats, as assessed by postoperative weight gain and rate and quality of wound healing. Results showed that the arginine requirement was increased by the wounding (unwounded young adult rats grew satisfactorily on the basal diet without arginine). Wounded rats did not grow unless supplemental arginine was given. Also, incisions of wounded rats ingesting the basal diet did not heal normally, e.g., wound strength was decreased.

2. In subsequent experiments, supplemental arginine hydrochloride given in the foood (0.5%) and drinking water (0.5%) to rats and mice ingesting a standard commercial rodent chow that contained 1.8% arginine, supported normal growth, reproduction, lactation, and longevity of normal rats was found to

   a. Accelerate wound healing in rats with dorsal skin incisions (breaking strenth) and with subcutaneously implanted polyvinyl alcohol sponges (reparative collagen accumulation).
   b. Lessen weight loss after injury.
   c. Not increase the gain in breaking strength of dorsal skin incisions and the accumulation of reparative collagen in subcutaneously implanted polyvinyl alcohol sponges in rats with 3^{o} burns involving 35% body surface or with bilateral femoral fractures.
   d. Increase thymic weight and the number of thymic lymphocytes in uninjured animals.
   e. Lessen the decrease in thymic weight and thymic lymphocytes of injured animals (femoral fractures, burns). (The thymotropic effect of dietary arginine on thymic size occurs without any effect on adrenal size. In this respect arginine differs from other thymotropic agents such as vitamin A, which prevents both thymic involution and adrenal enlargement after a variety of physical, chemical, or viral injuries).
   f. Increase the responsiveness of thymic small lymphocytes of uninjured rats to mitogens (phytohemagglutinin and concanavalin A).
   g, Restore toward normal the impaired responsiveness to these mitogens of thymocytes of injured (bilateral femoral fracture) rats.
   h. Increase the resistance of mice to a viral tumor (MSV) and a transplantable breast tumor (C3HBA).
   i. Accelerate allograft rejection.
   j. Be ineffective as a thymotropic or wound-stimulating agent in hypophysectomized rats maintained on ACTH, thyroxine, testosterone propionate, and with or without growth hormone; such effects require an intact pituitary-adrenal axis.
   k. Fail to increase wound healing/breaking strength of skin incisions in previously thymectomized rats; in the thymectomized rats the rate of gain of wound breaking strength was greater than in the non-thymectomized rats. When thymectomized rats were grafted with thymus, the rate of gain of wound breaking strength was less than in thymectomized rats but similar to that of normal rats. Arginine increased the rate of wound healing in thymectomized rats with thymus grafts, as it did in normal rats. The data suggest that the thymus is necessary for the positive effects of supplemental dietary arginine on wound healing.

l. Dessicated thymus fed orally to thymectomized rats ingesting a standard rat laboratory chow led to an increase in peripheral blood lymphocytes to the normal range. We believe this is due to the presence of low molecular weight polypeptides thymic hormones in the thymus which may be absorbed in functionally intact form, just as are certain other peptide hormones.

m. As mentioned in the section dealing with our studies of supplemental dietary vitamin A, when rats ingesting a standard rat chow diet are subjected to 3° burn involving 35% body surface area, there is an abrupt increase in urinary orotic acid which persists for the two weeks of study. When supplemental dietary arginine is added to the basal chow diet, there was little difference in urinary oritic acid during the first 6 days, but thereafter the level of urinary orotic acid excretion fell sharply in contrast to that of the rats ingesting the basal chow. This study was based on the well established oroticaciduria which occurs in arginine deficiency (insufficiency). These data support our hypothesis of increased arginine requirement after injury.

n. Decreases the weight loss, leukopenia, thymic involution and adrenal enlargement of mice subjected to 4.5 Gy whole-body-radiation.

Some relevant studies of others

1. Supplemental dietary arginine and glycine given to rats on a 25% casein diet lessens the urinary nitrogen excretion in those subjected to femoral fracture and/or ether anesthesia (Sitren and Fisher, 1977).

2. Further studies (Elsair et al., 1978) indicated that supplemental arginine diminishes the negative nitrogen balance of patients following major elective operations.

3. Supplemental arginine given to healthy men and women increases the in vitro mutogenic responsiveness of peripheral lymphocytes (Barbul et al., 1981).

4. Alexander and his colleagues (1980) found a high correlation between plasma arginine concentration and a number of parameters indicating resistance to infection.

b. Effects of Supplemental Dietary Ornithine

In other experiments, we have reported previously, that supplemental dietary ornithine given to rats and mice has the following effects:

(1) accelerates wound healing,
(2) it is thymotropic (thymic weight and number of thymic lymphocytes,
(3) lessens the thymic involution following the stress of partial body casting,
(4) increases the resistance of normal and stressed mice to MUSV-M inoculation and speeded the regression of those tumors which developed,
(5) accelerates skin allograft rejection,
(6) decreases the weight loss, leukopenia, thymic involution and adrenal enlargement of mice subjected to 4.5 Gy whole body-radiation.

All these effects of supplemental ornithine are like those of supplemental arginine, but it should be pointed out that dietary ornithine is not converted appreciably to arginine and it is possible that arginine may exert its actions via ornithine.

Study subsequent to our 1 November 1983 Progress Report

Supplemental Ornithine: Thymopoietic and Leukopoietic Acions in Irradiated Mice

Supplemental ornithine (1% of diet) enhances the anti-tumor effect of local irradiation (3000cGy) in C3HBA mammary tumors in mice. To examine ornithine-radiation interactions, 3 groups of 10 mice each were established; 2 of these received 450cGy whole-body gamma-irradiation. One of the irradiated groups and the unirradiated group were maintained on laboratory chow; whereas one of the irradiated groups was started on supplemental ornithine directly after irradiation. Ornithine prevented some of the radiation-induced weight loss and increased the rate of regain of lost weight. Animals were killed after 12 days. Radiation reduced thymic weight (44±1 mg vs 23± 1 mg) and the number of thymus cells ($32.5 \pm 0.5 \times 10^6$ cells/gland vs $12.3 \pm 7 \times 10^6$). In ornithine-fed irradiated group, these decreases were reduced by 50% in each case. In another similar experiment, white blood cell counts were made periodically in mice over a 30-day period, after which the animals were sacrificed for thymus analysis. As before, ornithine moderated radiation-induced thymic weight loss and cell numbers. Additionally, it also moderated circulating WBC decreases due to radiation. It is suggested that this thymopoietic and leukopoietic action of ornithine contributes to the enhancement of the anti-tumor effect of radiation by ornithine.

c. Discussion of Our Arginine and Ornithine Studies

There is evidence, then, that there is an increased dietary requirement for arginine after injury, so that while it is not a dietary essential amino acid for normal rats (growth, reproduction, longevity), it becomes so after injury as assessed by wound healing, nitrogen balance, thymus size, thymic lymphocytes, and resistance to certain neoplasms.

We think that the thymotropic and wound healing effects of arginine are due to nutritional mechanisms of arginine and influence of arginine on hormone-mediated reactions. Arginine administered intravenously is a secretagogue for growth hormone, glucagon, insulin, somatostatin, and pancreatic polypeptide and prolactin in several species. The effects of orally ingested arginine on pituitary or pancreatic hormone release have been studied in a very limited way. We had postulated that the difference in the route of administration may produce quantitative but not qualitative differences, since most of the ingested arginine is not degraded in the GI tract but absorbed as such and the rate of hepatic arginine degradation allows increases in systemic circulating levels of arginine. In this regard, it should be recalled that the route of administration, i.v. or oral, produces quantitative but not qualitative differences for non-hydrolyzable agents involved in stimulating secretion of other hormones, e.g., glucose or tolbutamide for insulin. Recently it has been reported that arginine and lysine ingested together, each in a low dose, is a growth hormone secretagogue in humans, neither alone in this same low dosage did. The dose of arginine used was much less than that used in the usual i.v. arginine secretagogue test (91).

We have conducted a number of experiments to determine whether the secretagogue action of arginine on growth hormone may be important for the effects of supplemental arginine on wound healing. Growth hormone administration to burned patients has been shown by Wilmore and associates (92) to decrease urinary nitrogen excretion after injury.

Growth hormone is also known to stimulate fibroplasia under certain conditions. Direct stimulation of cultured rat fibroblasts by rat growth hormone has been demonstrated by Moon and St. Vincent (93) and Murakawa and Raben (94) showed an increase in DNA synthesis in rat cartilage when growth hormone was added *in vitro*. Wettenhall *et al.* (95) found stimulation of proline and hydroxyproline incorporation in rat bone organ culture by insulin but not by growth hormone added *in vitro*, and concluded that growth hormone action may be mediated by insulin rather than being directed. There is thus still no agreement on whether growth hormone can act directly on fibroblasts in culture; *in vivo* there is considerable evidence that growth hormones exerts many of its effects through "sulfation factor" activation (somatomedin) (96) (97).

Our own results confirm that human growth hormone added *in vitro* to human fibroblast lines strongly stimulated DNA and protein synthesis (98).

Closure of healing open wounds was found to be stimulated by growth hormone in rats (99) but another study of small skin open wounds in human volunteers found no such stimulation (100). In the latter study, however, growth hormone was administered starting the 6th day after wounding so that an early phase of vigorous cell proliferation had already passed by the time at which the hormone was given.

Some additional recent animal studies have failed to clarify the effect on wound healing of supplemental growth hormone given to animals with intact CNS-hypothalmus-pituitary systems (101, 102).

As in the case of the results of our wound healing studies, we believe that part of the thymotropic effect of arginine may be related to its growth hormone secretagogue effect. It is known that growth hormone increases thymic size, stimulated thymic DNA, RNA, and protein synthesis, and antibody response of hypophysectomized rats to sheep erythrocytes; conversely, the thymus atrophies after hypophysectomy or after administration of anti-growth hormone serum.

Further Discussion of the Role of Arginine

The data from our experiments briefly enumerated above indicate that the arginine content of the chow (1.8%), while adequate for growth, reproduction, and longevity of the uninjured rat, is inadequate for wound healing and maintenance of thymic weight and thymic lymphocytes after an injury of moderate severity. Arginine also has important anti-tumor actions. In considering why these effects should occur, it should be recalled that arginine has many biological actions:

(1) It is a constituent of many proteins, including nucleoproteins and collagen.
(2) Arginine is a precursor for many amino acids, such as ornithine, glutamic semi-aldehyde, glutamic acid and proline (and thus also of hydroxyproline).
(3) Arginine may act as a precursor for energy-rich phosphoguanidines, such as creatine phosphate.
(4) Arginine influences various enzymatic activities; thus, in the urea cycle, arginine leads to increased ureagenesis by being a positive effector of ornithine trans-carbamylase; on the other hand, within the same cycle, arginine is a negative effector of N-acetyl glutamate synthetase, thus inhibiting ornithine synthesis from glutamate when arginine is available as a precursor.
(5) Some of the effects of supplemental arginine and ornithine may be mediated as a result of secretagogue actions, notably on growth hormone, but, also, on insulin, glucagon, pancreatic polypeptides, somatostatin and prolactin, or they may act to inhibit the release of ACTH. (This aspect of arginine's action is one we have emphasized in a number of our experiments; see Progress Report Appendix for further discussion of this matter)
(6) Arginine may influence inflammation, neovascularization and fibroplasia by being one of the metabolic precursors for diamines (putrescine, agmatine) or polyamines (spermine, spermidine); for example, ornithine decarboxylase activity increases greatly just before the cellular division of liver regeneration following partial hepatectomy.

The relationships mentioned in 4), 5) and 6) are complex.

Arginine's position and role among dietary amino acids has been equivocal. The classic studies of Rose and his co-workers (103) showed that nine amino acids are dietary essentials for growing rats. These amino acids were leusine, isoleucine, valine, phenylalanine, threonine, methionine, histidine, tryptophan and lysine. Absence of any of these was marked by failure to grow, weight loss and death. Lack of arginine alone resulted only in lesser rates of growth (similar to that we later observe in group 1, experiment 1), causing Borman et al. (104) to conclude that arginine was the least essential of the amino acids necessary for growth. Subsequently it was shown that dietary arginine prevents excessive urinary losses of metabolites such as orotic acid and citric acid in young rats (105).

Rose (106)extended his rat sudies by examining the dietary amino acid requirements of young healthy men, starting with the nine amino acids which he had shown to be essential for the growing rat. In addition, he studied the arginine requirement of these human subjects. Single amino acids were removed from the diet and the effects on nitrogen balance and weight maintenance over a ten day period were measured. In these studies, arginine and histidine were found to be dispensable. Later, however, histidine was shown by others to be essential for nitrogen balance and erythropoiesis for both normal and chronically uremic men (107).

At the cellular level, all of the usual amino acids found in proteins are necessary for cell multiplication and protein synthesis, including the synthesis of enzymes and other proteins involved in the healing process. In this context, all amino acids are essential; the term "essential", as commonly used, refers to a dietary requirement for the specific amino acid. All of the dietary essential amino acids (other than lysine and threonine) can be synthesized by mammals, including humans. However, precursors such as the corresponding alpha keto- or alpha hydroxy-analogs of the essential amino acids are not normally available in amounts that allow synthesis of these amino acids in adequate quantities (108 - 110), particularly under conditions of increased metabolic activity. When the requirement for an amino acid such as arginine is increased following injury, a previously "non-essential" dietary amino acid becomes "essential."

The role of dietary arginine in collagen synthesis is not clear. The studies of Zinker and Rojkind (111)on collagen synthesis in chick bone culture and that of Dunn and Rojkind with damaged livers (112)showed that arginine was a more effective metabolic precursor for proline and hydroxyproline in newly synthesized collagen than glutamate. On the other hand, Shen and Strecker (113) showed that arginine was less effective than glutamic acid in promoting collagen synthesis in human fibroblast cultures. Our results are consistent with the findings of Zinker and Rojkind and Dunn and Rojkind.

Arginine may stimulate collagen synthesis in conditions where glutamic acid is no longer limiting, because arginine's uptake and transport is independent of glutamic acid transport. An alternative explanation is that in repair systems, the glutamic acid reduction to glutamic semialdehyde is already proceding maximally and that transamination of ornithine to yield glutamic semialdehyde opens up an alternative route to meet the increased demand for proline in reparative collagen synthesis.

$$\text{glutamic acid} \xrightarrow{\text{reduction}} \text{glutamic semialdehyde} \longrightarrow \text{proline}$$

$$\text{arginine} \longrightarrow \text{ornithine}$$

In our last application, we advanced the view developed by one of us (E. Seifter) that ornithine is the mediator for some of the responses observed when arginine is given and that in these instances arginine serves mainly as a way of delivering ornithine to appropriate cells. This hypothesis states:

$$\text{Arginine} \atop \text{Ornithine} \left(\begin{matrix}\text{growth hormone} \\ \text{secretagogues}\end{matrix}\right) \rightarrow \text{growth hormone} \longrightarrow \text{somatomedin} \rightarrow \text{ornithine decarboxylase induction}$$

$$\text{Injury} \downarrow \text{ornithine decarboxylase (ODC)}$$

cell activation ⟶ cell multiplication ⟶ regeneration + repair

ODC

ornithine ⟶ putrescine ⟶ polyamines ⟶ protein synthesis, regeneration, repair

↓

inhibition of ODC induction

These reactions underlie both the systemic and local effects of arginine in wound healing. The unique properties of arginine and ornithine are:

(1) They can elicit systemic metabolic responses that result from the growth hormone (other hormones as well) released due to the action of these basic amino acids. In order for growth hormone to exert some of its anabolic actions it must be taken in by organs that can produce somatomedin or insulin-like substances in response to growth hormone(114-115). Arginine and ornithine may play a role in the reaction growth hormone (GH) ⟶ somatomedin. Suggestion for support of this view derives from the finding that while plasma GH may be elevated in trauma, somatomedin is not(116). During this same period plasma arginine drops (117,118) as (a) the liver increases urea production and (b) kidney decreases arginine synthesis.

Arg, Orn

↘

Injury ⟶ GH ⟶ arginine / ornithine ⟶ somatomedin

↓

cell activation + ODC induction

Arg ⟶ ornithine ⟶ putrescine ⟶ polyamines, cell proliferation protein synthesis

Thus a second role for arginine and ornithine is to provide the substrate for the induced ODC.

At the local level, injury ⟶ release of local factors (e.g. prostaglandins) ⟶

↓

ODC induction

ODC + arg, ornithine ⟶ putrescine

Putrescine ⟶ polyamines, reparative growth

↓

inhibition of ODC induction

Thus arg + ornithine can exhibit strong regulatory functions on cell proliferation and protein synthesis.

1. Increase rate of somatomedin synthesis (hypothesis)
2. Increase rate of putrescine synthesis
3. Act as substrate for ODC induction
4. Antagonize the negative feedback of putrescine in ODC induction
5. Possible reinforcement of local tissue reaction

References

Discussion of Our Arginine and Ornithine Studies and

Further Discussion of the Role of Arginine

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II. Methods to Increase Fibroblast Proliferation and Collagen Synthesis in Wounds. Implantation of Cultured Fibroblasts into Animals

The critical role fibroblasts play in wound healing has long been recognized. Without adequate numbers of fibroblasts, without properly functioning fibroblasts, wound healing cannot proceed. These cells, among their special functions, are the sole manufacturers of collagen, and the synthesizers of some of the essential components of the ground substance. The adverse effects of "too much" cortisone on wound healing, for example, are due in large measure to a substantial decrease in the numbers of fibroblasts due in part to inhibition of their multiplication and some changes in their ultrastructure. In ascorbic acid deficiency, there is no decrease in the number of wound fibroblasts, nor in the rate in which they appear, but there is a significant change in the ribosomal arrangement along with the endoplasmic reticulum, a disarrangement which is promptly restored to the normal orderly "polysomal" arrangement when ascorbic acid is given - Ross and Benditt.

Fibroblasts have generally been considered to begin making collagen only after the "growth" phase but recent results from our laboratory indicate that fibroblasts in the logarithmic growth phase can synthesize and extrude collagen.

The rates at which the entrance and multiplication of fibroblasts enter the wound under normal circumstances are well known. These rates are slow enough to have encouraged investigators to attempt to accelerate them, with the expectation that wound healing would be speeded up thereby. Prudden has reported limited success in this regard with cartilage powder and n-acetyl-glucosamine polymers applied locally to the wound.

Our objectives in this phase of our studies were to determine whether cultured diploid fibroblasts survive when added to polyvinyl alcohol sponge implants in rats, remain viable upon reincubation, accelerate the synthesis of ground substance and collagen, and increase the rate of healing and the strength of wounds. The long term fate of implanted cultured diploid fibroblasts was also studied, with particular attention to possible tumor formation.

A. Background

The survival of and the reactions to reimplanted cultured cells in animals or patients have been studied by a number of investigators. Most of these studies were, however, concerned with the investigation of rejection phenomena and/or of specific tumor antigens. Southam *et al.* showed that human and other neoplastic cell lines grew better after subcutaneous injection into cancer patients than in healthy volunteers; chick embryo fibroblasts did not survive Established epithelial cell lines from normal tissues also survived well after subcutaneous injection into cancer patients, but such established lines at the time of implantation were no longer diploid (Moore *et al.*, 1956). Cells of the human epidermal line NCTC 3075 survived for several months in patients with advanced *mycosis fungoides* but for shorter time periods in patients in less advanced stages of the disease, indicating an impairment of the immunological defense mechanism (Van Scott *et al.* (1956). In these studies, the implanted epithelial cells did not keratinize or otherwise carry out specialized functions of differentiated cells. Billingham

and Silvers (1970) found that pigmented cells of guinea pig cells survived well when transferred into non-pigmented areas within the same animal or a sibling within a highly inbred strain. Human skin epithelial cells grown in culture can be successfully autotransplanted to burned patients: the long term fate of these transplants has not been determined, i.e., whether neoplasms may develop.

B. Tissue Culture of Fibroblasts

Animals of highly inbred strains (Fischer rats) were used in order to obviate genetic variability.

Fibroblasts cultures were derived from the skins of newborn animals by mincing and trypsinization (0.1 - 0.25% trypsin, at times in the presence of 0.001 - 0.002 M NA2 EDTA). The cultures were grown in Petri dishes in Eagle's MEM with 5 - 10% calf serum and subcultured until enough material for the implantation experiments was available. For the experiments, the cells were dispersed by trypsinization, washed repeatedly with balanced salt solution to remove calf serum proteins, and finally suspended in balanced salt solution for reinjection into experimental animals. Aliquots of the suspension were also transferred into Petri dishes with the standard growth medium in order to determine the viability of the suspended cells at the time of reinjection.

C. Determination of Survival and Function of the Cultured Fibroblasts When Injected into Animals; Effect on Wound Healing

In our investigations the effect of the implantation of Fischer rat fibroblasts grown in culture on a) the rate of collagen synthesis in subcutaneously implanted "Ivalon" polyvinyl alcohol sponges and b) the healing of skin incisions was studied in rats.

Rat embryo skin fibroblasts were derived from the dorsal skin of 15-17 day rat embryos. Fibroblasts from reparative tissue were obtained from polyvinyl alcohol sponges subcutaneously implanted into rats and removed after 4-5 days. Dulbecco-Vogt medium containing 0.024 M of HEPES buffer (pH 7.35) and supplemented with 10% fetal calf serum, penicillin, streptomycin. amphotericin B, and aureomycin, at 36°-37°C in 10% CO_2/air, were the conditions of cell culture.

The cells were harvested by trypsinization. washed twice by centrifugation in cold serum-free culture medium, and were then resuspended in serum-free medium ($1-2 \times 10^7$ cell/ml). 0.1-0.2 ml of the cell suspension was instilled into each polyvinyl alcohol sponge. Control sponges containing an equal volume of serum-free tissue culture medium only. The sponges (4 per animal) were inserted aseptically into 250 g rats under pentobarbital anesthesia in subcutaneous pockets. The recipient rats were the same inbred Fischer strain from which the cultured fibroblasts had been derived.

In sponges removed four days after implantation, a significant increase in collagen content after inoculation with repair tissue fibroblasts was observed although even in the fibroblast-inoculated sponges the collagen content was low at this time. After seven days, the collagen content of the fibroblast-inoculated sponges was more than twice as high as in the controls. In fact, the amount of collagen present after 7 days in fibroblast-inoculated sponges was approximately the same as that in non-inoculated sponges after 14 days. The increase was about equal when skin fibroblasts and when fibroblasts from repair tissue were inoculated or, in one experiment, from human lung (WI-38).

Preliminary experiments, in which rapidly proliferating fibroblasts were tagged by pulse-labelling and were then instilled into sponges prior to implantation into rats, showed that 10 - 15% of the radioactive label was present in the sponges at the time of removal one week later. This radioactivity was presumably present in viable cells since dead cells and cell debris would likely have been removed from the tissue within this time period. The fibroblasts in the inoculum, although only a fraction of them may remain viable, are thus sufficient to provide a significant acceleration of collagen synthesis during early stages of wound repair.

In other experiments, cultured rat fibroblasts, immobilized in plasma clots, were introduced into dorsal skin incisions in rats to determine whether wound breaking strength would be affected by the instillation into the wound of collagen synthesizing fibroblasts at the time of wounding. We believe that there was a mechanical problem in maintaining the instilled fibroblasts at the wound site, since the skin of the rat is relatively thin and it is certain that most of the instilled fibroblasts do not stay in the wound itself but are distributed in the underlying tissue. Despite this, in two of three experiments in which cultured fibroblasts derived from reparative tissue formed in implanted polyvinyl alcohol sponges were instilled into the incision at the time of wounding and immobilized in a plasma clot produced by the simultaneous instillation of a small amount of plasma and thrombin, there were statistically significant ($p < 0.01$), though modest (10-20%) increases in wound breaking strength, 12 days postoperatively. However, in only one of seven other experiments with cultured fibroblasts of other types (e.g., derived from the skin of rat embryos) was an accelerating effect on the gain of wound strength seen. These data suggest that there may be something special about fibroblasts derived from actively repairing tissues.

D. Long-term Effects of Cultured Fibroblasts, with Emphasis on Possible Tumor Formation

a. Background

The capacity of culture-grown cells derived from malignant tumors to cause tumors when re-injected into animals has been studied by a number of investigators. Southam, Moore and Rhoads established that human established cell lines originating from malignant tumors survived in terminal cancer patients, forming nodules which persisted up to several weeks in cancer patients but for much shorter periods in normal subjects. Similar results were reported by Nadler and Moore who also injected human malignant tumor cells into cancer patients and found local persistence of the cells for several weeks but no tumor propagation and no systemic reaction.

Earle and his associates studied by in vitro cultures and injection technique possible changes in the morphology of fibroblasts derived from normal mice and their ability to lead to tumor formation when injected (after culture) into mice, particularly when the cells were cultured in the presence of a carcinogen (20-methylcholanthrene). They presented in 1943 the results of injection of seven strains of fibroblast cultures into young C3H mice; these strains had all been derived originally from a primary culture of fibroblasts of a C3H mouse. Included were two control strains (i.e., no methylcholanthrene added knowingly to the cultures), one of which was altered very slightly morphologically during successive passages in culture while the other was more altered. The cells grown in the presence of methylcholanthrene showed substantial morphologic changes. Cultures of each cell strain on reinjection into C3H mice gave rise to tumors. The alterations in the control cultures were thought by Earle and associates to have probably having resulted from trace contamination of the control cultures with 20-methylchloranthrene, although the possibility of the changes having arisen spontaneously could not be ruled out. These various strains of cells were kept growing by Earle and associates for many years, but the methylcholanthrene was removed from the culture media. As the years went by, those cultures of cells originally treated with methylchloranthrene led to fewer tumors

when injected into mice, but the control strain which had led to the highest incidence of tumors when injected continued to maintain this property (20).

Aaronson and Todaro observed that cells of established mouse embryo **lines formed tumors when reinjected in mice. The cell lines used were all derived from a single pool of 14 to 17 day old Balb/c mouse embryos. "Balb/c mouse embryo lines maintained in culture for over 200 generations under conditions that minimize cell-cell contact do not become tumorigenic but lines cultivated under conditions where there is extensive cell-cell contact become tumor-producing within 30 generations. The tissue-culture property that correlates best with tumorigenicity is the loss of contact inhibitions of cell divisions".**

Southam, Moore and Rhoads stated that normal diploid human embryonic fibroblasts did not produce tumors in any of their experiments, and indeed all the cell lines that were found to produce tumors in the above experiments were heteroploid.

These findings were confirmed more recently by Petricciani et al. who tested the tumorigenicity of a large number of cell lines in primates treated with antithymocytic globulin. In these animals, whose immune rejection system was inhibited, none of the non-transformed diploid cell lines, or primary cell lines, or primary cell explants, including W138, chick embryo and duck embryo fibroblasts produced tumors, while established cell lines or virus-transformed cells were almost 100% tumorigenic.

b. Our long-term experiments - Long-term studies were carried out in which the possible causation of tumors by the re-implanted fibroblasts was monitored. Culture-grown rat fibroblasts (2-3 x 10^6 per site) were injected subcutaneously into young rats while in another group of rats polyvinyl alcohol sponges, inoculated with 2-3 x 10^6 cell each, were inserted subcutaneously; control rats for the latter received the sponges, but no cells; control rats for the former received only the media in which the cells were suspended.

In these studies, a suspension of highly inbred Fischer rat fibroblasts cells derived from the reparative tissue of polyvinyl alcohol sponge implants and grown in tissue culture (15 subcultures over a 2 month period) was added to sponge implants in other Fischer rats of the same highly-inbred strain and allowed to remain implanted for a year or more. When examined just before planting, the cultures of cells pooled appeared unchanged morphologically after 15 passages. For six months, these rats grew well and no abnormal signs were noted. Palpable tumors at the side of the sponge implants with cell suspension were found in 4 of 10 rats after seven months. Eventually all of the rats developed massive tumors on the side with the sponge implant containing added cell suspension; no tumors developed in the other side of the rats with sponge implants containing added media only.

Small pieces (1x1 mm) of viable tumor (fibrosarcoma, histologically) were excised and implanted subcutaneously in rats; all rats developed tumors by two weeks after the injection. A suspension of tumor cells was prepared by grinding pieces of viable tumor tissue suspended in Dulbecco's culture medium in a glass Potter-tissue homogenizer and the suspension injected subcutaneously in other rats. For 4-5 weeks, no palpable tumors were noted but during the next month, all (5 out of 5) rats developed tumor sites where the suspension was injected.

In other rats, a 0.22 μ Millipore filtrate of the cell suspension of viable piece of tumor tissue was injected subcutaneously. Over a 12 month period, these rats grew normally and showed no signs of tumor formation.

This study was repeated in other Fischer rats with sponge implants containing fibroblasts derived from added wound sponge fibroblasts. These cell cultures were examined just before plating and appeared morphologically unchanged after 8 subcultures. For the past five months, these rats grew normally with no untoward signs of abnormalities, but by 12 months all had developed tumors, which were readily transplanted to other rats.

Conclusion

In view of these findings, it would appear that if one is to attempt to speed wound healing by the implantation of cultured fibroblasts, only "young" cultures must be used, before any possible transformation has occurred. These findings make us concerned about this current use by others of skin epithelial cells grown in culture for many generations and then used as skin transplants in burn patients, especially children. The possibility of late neoplasia cannot be ignored.

(continued next page)

III. Methods to Increase the Rate at which Wounds Gain Strength by Controlling the Rate of Cross-linking of Reparative Collagen

A. Background

We pointed out in our previous Research Progress Reports that the breaking strength of wounds is largely dependent not only on the amount of collagen present and its architectural arrangement at the gross and light microscopic levels, but also on the degree of the cross-linking at the intra-and inter-molecular levels and on the associated ground substance components. Cross-linking of reparative collagen begins when collagen is synthesized by the fibroblasts and secreted or extruded into the extracellular space and continues at a rapid rate for many weeks, following which it slows, but continues possibly throughout the life of the animal. The importance of the intra- and inter-molecular bonding of collagen on the strength of the collagen and thereby of the wound breaking strength is illustrated by the "negative" effects of administered lathyrogens (such as B-amino propionitrile, penicillamine, penicillin, and isonicotinic acid hydrazide) on the healing wound.

Normally, it takes a number of weeks for the major portion of cross-linking of newly formed wound collagen to occur. This can be accelerated in vitro, e.g., by immersing the wound in 10% buffered formalin for 48-72 hours. (Figs. 4 - 5). We are aware of the possibility that an increase in the rate of cross-linking of the reparative collagen may influence the rate of collagenolysis and the remodelling process. We hypothesized that one whould be able to bring this about in vivo. How to do this, and how to limit the effects to the wound site, is the challenge. Our aim is to reach a relatively "mature" degree of collagen cross-linking soon after the newly synthesized reparative collagen is laid down in the wound without affecting collagen cross-linking elsewhere in the body (because of the postulated relationships of increasing collagen cross-linking to aging) and without interfering with the architectural rearrangement of collagen fibrils and fibers which goes on in the healing wound.

In some studies of the healing of rat skin incisions, we showed that breaking strengths of normal healing wounds increase after fixation in formaldehyde at every point postoperatively during the first year (which was as long as the experiment was continued) (1). The absolute increase in wound breaking strength induced by formaldehyde increases with increasing age of the wound until approximately the 7th postoperative week after which this difference remains constant. This sharp breaking point corresponds with the sudden decline, also about the 6th to 7th postoperative week, in the rate of collagen increase in the wound as judged histologically and in the gain of the fresh wound strength. The percentage difference in breaking strength between fresh and formaldehyde fixed wounds also shows a sharp break point at about the 7th week, but this curve is the mirror image of the absolute difference curve; that is, there is a rapidly declining percentage effect of formaldehyde until the 7th postoperative week and then a leveling off (Fig. 6). The data demonstrate that the effect of formaldehyde is substantially greater on an immature wound than on a mature one. In brief, the earlier, sparser, and finer collagen fibrils lend themselves to a greater number of cross-links per macromolecule after formaldehyde treatment than do the later coarser fibrils.

We planned to take advantage of the special characteristics of the healing area (e.g., the very rapid formation of collagen which is not yet cross-linked to a substantial degree and the pH of the healing tissue) in our attempts to accomplish this.

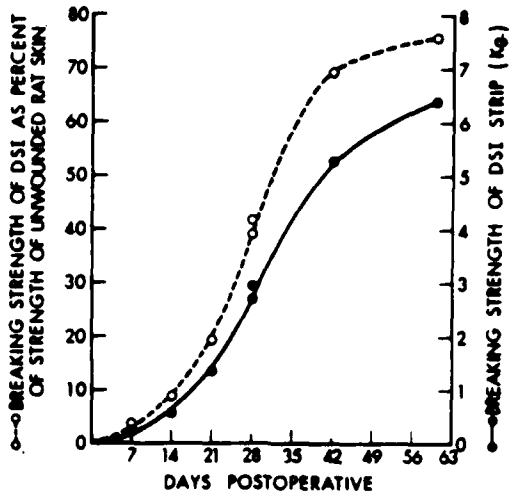

FIG. 4. Increase in breaking strength of a healing wound shown absolutely and as per cent of strength of comparable unwounded skin.

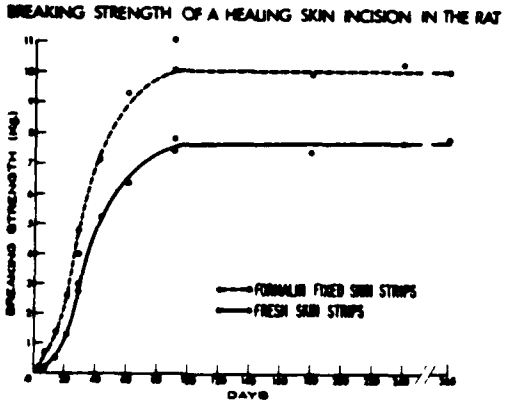

FIG. 5. Simple plot of breaking strength of a healing skin incision in rat as a function of healing time.

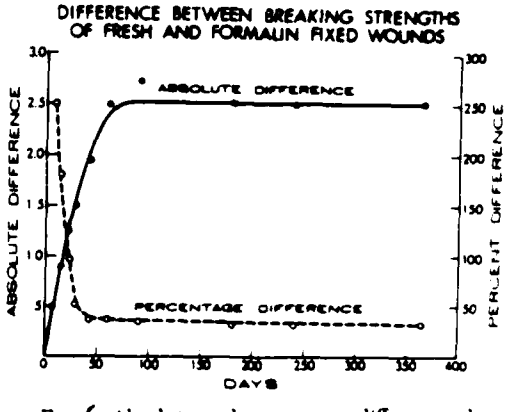

FIG. 6. Absolute and percentage differences between breaking strengths of fresh and formalin-fixed wounds as a function of time of healing.

B. Our Early Studies Prior to Award of Army Research Grant

Most of the experimental animal studies carried out by other investigators and ourselves prior to 1969 in which the rate of collagen cross-linking has been modified have dealt with its interference and impaired wound healing. These studies have helped elucidate the nature of collagen cross-linking and the mechanisms by which impairment is brought about by specific agents, such as β-aminoproprionitrile, penicillamine, penicillin and isonicotinic acid hydrazide.

To briefly summarize some of our findings in this regard are:

(1) We showed that the very weak wounds of animals treated with BAPN increased remarkably after *in vitro* treatment with formalin, so much so that the wounds attained the same strength as formalin fixed wounds of normal animals. This, together with the histologic data and quantitative measurements of hydroxyproline in the wound sponge granuloma, showed that there was no failure of collagen synthesis, but a failure of cross-linking. These observations demonstrated the significance of testing the breaking strength of incisions in the fresh state and after formaldehyde fixation as a measure of collagen cross-linking, a matter which Levenson and his associates at Walter Reed Army Institute of Research had first shown for healing incisions in normal rats and which we have since confirmed in a number of other experimental situations.

(2) We showed that impaired wound healing due to penicillamine was due to primarily impaired cross-linking and not to a combination of impaired collagen synthesis and cross-linking as originally suggested by others.

(3) One of us, Eli Seifter, suggested that the main action of penicillamine was to interfere with the cross-linking of collagen aldehydes by the formation of thiazolidine rings, rather than to its action as a copper sequestrant. The findings of various groups (Piez, Minmi, Tanzer, Jaffe) have shown in well-conceived experimental ways what Seifter had deduced from wound healing data regarding the mode of action of penicillamine.

(4) We showed that oral penicillin in high doses acts as a lathyrogenic agent probably because of its conversion to penicillamine, penicillic acid and other such metabolites.

(5) We showed in experimental studies utilizing an aldehyde-staining histologic technique that the effect of isonicotinic acid hydrazide in interfering with collagen cross-linking is after the aldehyde formation stage.

C. Studies Conducted with Army Research Support

Beginning with the Award of our Army Medical Research and Development Research Contract in 1969, we began a shift in emphasis of our work in the collagen cross-linking wound healing area. Our investigations in this area have aimed at devising systemic and local methods for producing stronger wounds at earlier times in the healing process and thereby shorten convalescence.

We have been concerned primarily with ways of increasing cross-linking of reparative collagen and thereby wound strength by:

1. the use of enzymes and coenzymes in the wound,

2. the use of chemicals which will cause by non-enzymatic means the aggregation and cross-linking of reparative collagen *in situ*.

a. Use of Enzymes and Co-enzymes

The cross-linking of collagen involves the enzymatic conversion of certain E-amino groups of specific lysyl residues of collagen to the corresponding aldehydes. These aldehydes cross-link by one of two main reactions. Either they form aldimines with E-amino groups on neighboring chains or they form aldol links with other aldehydes. It should be noted that in the early phase of formation of reparative collagen some of the cross-links are reversible, i.e., the Schiff's base has not yet been reduced.

A number of years ago we undertook pilot experiments with the aim of isolating an enzyme from sponge granulomas and wound fluid which could cross-link lathyritic collagen. A crude enzyme preparation was obtained which had the properties of increasing the viscosity of collagen and of altering the gelation properties of lathyritic collagen. Our aim was to treat wounds with this enzyme for the purpose of strengthening the wound.

Subsequent to this, others have shown that cross-linking of collagen is indeed mediated by extracellular enzymes. It is well established that collagen achieves its mature form as an extracellular protein. It is also clear from the work of a number of investigators that collagen is extruded from the fibroblasts before the stage where all the lysyl residues have been oxidized to the corresponding aldehyde, and before cross-linking occurred. Thus, extracellular aldehyde-poor collagen accumulates in BAPN treated rats and aldehyde-rich collagen accumulates in penicillamine treated rats. A reasonable inference, then was that the cross-linking of collagen is mediated by extracellular enzymes, and this has been demonstrated.

Amine oxidase had earlier been implicated previously in collagen cross-linking. Thus, based upon the correlation between the lathyrogenic action of certain compounds and their inhibition of diamine oxidase (DAO) activity, several groups, e.g., Tanzer, J. Cell Biol. 22: 623 , 1962), Levene (J. Exper. Med. 116: 119, 1962), Page and Benditt (P.S.E.B.M. 124:454 , 1967), and ourselves had suggested that lathyrogens of the BAPN type act by inhibiting extra-cellular DAO. We had further suggested that DAO probably acts in strengthening salt-insoluble as well as salt-soluble collagen.

The work of Siegel and Martin (J.B.C. 245: 1653, 1970) and Fowler and Miller (B.B.R.C. 40: 1970) made it clear that enzymes derived from normal bone and cartilage: a) can oxidize selected lysyl amino groups of normal and lathyritic collagen to the aldehyde stage, b) produce an increase in the molecular weight of these collagens via a cross-linking mechanism. The enzyme is a copper protein and requires pyridoxal phosphate. It has many properties of the amine oxidases but differs from the serum amine oxidase in its substrate specificity. Thus lysyl oxidase is a special example of the class of proteins known as amine oxidases.

1. We have report previously that injections into wounds of an extract of chick embryo bones prepared by the method of Siegel and Martin noted above:

(a) increased the breaking strength of healing incisions of normal rats tested in the fresh state ($p < 0.001$),

(b) did not increase the strength of the incisions measured after formalin fixation,

(c) lowered the ratio of the breaking strengths of the healing incisions of normal rats tested after fixation in formalin to the breaking strengths tested in the fresh state ($p < 0.005$),

(d) increased the breaking strengths of healing incisions tested in the fresh state of animals receiving INH which we have shown interferes with normal

cross-linking of collagen ($p < 0.001$) and lowered the ratio of the breaking strengths of the healing incisions of the INH-treated rats tested after formalin fixation to the breaking strengths tested in the fresh state ($p < 0.001$),

(e) led to similar results in one of two experiments with BAPN-treated rats (1.2-1.5 g BAPN/kg diet), (BAPN interferes with normal cross-linking of collagen to a greater extent than INH; BAPN interferes directly with the activity of lysyl oxidase), and

(f) did not change the hydroxyproline content of reparative tissue in s.c. implanted polyvinyl alcohol sponges of normal rats. Heated chick embryo bone extract preparation did not lead to the above effects.

These data support our hypothesis that injections of the chick embryo bone extract increases the rate of cross-linking of reparative collagen.

2. We found also that the instillationof B-NADH alone did not increase the breaking strength of the skin incisions tested in the fresh state to the same extent as did lysyl oxidase. When B-NADH and lysyl oxidase were both instilled into the healing skin incisions, the increase in fresh breaking strength was significantly greater than when the lysyl oxidase alone was injected. The breaking strengths of the formalin-fixed strips were similar in all groups,supporting the view that the action of B-NADH in potentiating the effect of the lysyl oxidase was to increase the rate of cross-linking of the reparative collagen.

3. Diamine oxidase (DAO) (porcine, commercially available) is not the natural occurring collagen cross-linking enzyme, but its action on amine groups resembles in some ways that of lysyl oxidase on amino groups. We have found in several experiments that the injection of diamine oxidase into healing dorsal skin incisions of the rats increases the rate of gain of wound strength in a way similar to that induced by the injection of lysyl oxidase, that is, an increase in wound breaking strength when measured in the fresh state but not after formalin fixation. These data are consistent with an increase in reparative collagen cross-linking; a possible mechanism for this involves diamine oxidase acting on polyamines present in the wound causing the liberation of aldehydes which then cross-link collagen. An analogy to this is to be found in the cross-linking of fibrin by the action of platelet derived enzymes on fibrin and polyamines. It is possible that the DAO will also influence fibroplastic proliferation and collagen synthesis, since certain metabolites which influence cell division, such as spermine and spermidine, may act as substrates for DAO.

b. Use of Non-enzymatic Chemicals

Experiments with formaldehyde liberator chemicals were carried out. As mentioned, formaldehyde's cross-linking effect on collagen is due to the formation of methylene bridges between the amino groups of lysine and other adjacent amino groups substituted methylene groups. The toxicity of formaldehyde precludes its use as such in vivo but we think it may be possible to use chemicals which will react locally in the wound to yield formaldehyde locally with little systemic effect on collagen elsewhere, and without local or systemic toxicity. We believe that the local application of formaldehyde liberators is more apt to provide the desired local effect on increasing the rate of cross-linking or reparative collagen than systemic administration.

Hexamethylene tetramine releases formaldehyde slowly. We have conducted several experiments in which hexamethylene tetramine in various concentrations (0.5-15 %) was injected daily along the length of our standard 7 cm dorsal skin incision of rats, beginning a few days after operation (so as not to alter the early inflammatory response, and continued until the rats were sacrificed a number of days later, i.e., the hexamethylene tetramine was injected during the period when the rate of spontaneous cross-linking of collagen is occurring at its maximal rate. In no case did the hexamethylene tetramine affect the breaking strengths of the skin incision tested in the fresh state or after fixation in 10% buffered formalin. The negative effects of these experiments may reflect the fact that the hexamethylene tetramine was injected

as a saline solution, and may have been absorbed very rapidly before it could induce cross-linking of the reparative collagen.

IV. Studies with Supplemental Dietary Vitamin E

A. Supplemental Vitamin E Slows Allograft Skin Rejection

Although vitamin E (DL-alpha-tocopherol) has been used by clinicians (and the lay public)to inhibit some inflammatory and hyper-immune reactions, laboratory data to support either use is limited. Since allograft skin rejection is enhanced by inflammatory and cell-mediated immune reactions, we felt that this system would be useful in assessing vitamin E's cell-mediated immune activity in vivo. Because we had shown previously in rodents that supplemental vitamin E, like some other anti-inflammatory immune suppressive agents, causes thymic involution, we postulated that supplemental vitamin E would prolong allograft skin surfival.

Three experiments were conducted using the test system of Medawar and Billingham. In each experiment, healthy six-month old C3H/HeJ mice, were divided randomly into two groups; one group was fed Purina laboratory chow (containing 65 mg vitamin E, about 4X the NRC RDA of vitamin E for mice) for 3 days: the other group was fed the same chow supplemented with vitamin E as shown in the table below. All drank tap water and ate ad libitum throughout the experiment. On the 4th day, all mice were subjected to excision of full thickness dorsal skin (diam = 1 cm) and all received C57Bl/6J skin transplants. Fast-setting Paster of Paris body casts were used to secure the graft. Each group of mice continued on its preoperative diet. Casts were removed on the 7th post-grafting day.

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Data regarding the times of skin allograft rejection are shown below:

Table

	Expt. 1 (200mg/kg diet)			Expt. 2 (800mg/kg diet)			Expt. 3 (2g/kg diet)		
	Chow	Vit. E	P	Chow	Vit. E	P	Chow	Vit. E	P
Days of Rejection	9.8±0.3	11.5±0.4	< 0.01	10.1±0.5	12.6±0.5	< 0.001	10.2±0.4	11.9±0.2	< 0.00

Thus, vitamin E in amounts 13X RDA increased skin allograft survival time moderatly, but significantly. Larger intakes (but still non-toxic) of vitamin E had no further effect.

In Experiment 1, the mice were killed on the 16th day and their thymuses examined. Thymus size was 28.8±1.3 mg for control mice versus 10.0±2.0 mg for the vitamin E supplemented (200 mg/kg diet) mice ($p < 0.001$); the number of thymus lymphocytes was 12.8±1.8 millions for control mice versus 1.1±0.9 millions for vitamin E supplemented mice ($p < 0.001$).

In conclusion, the experimental data show that supplemental vitamin E prolongs skin allograft survival and that this is likely consequent to the observed thymic involution. This is in sharp contrast to our previously reported findings with supplemental vitamin A; supplemental vitamin A increases thymic size and number of thymic lymphocytes and accelerates skin allograft rejection.

B. Supplemental Vitamin E Lessens Peritoneal Adhesion Formation

We have previously shown that dietary supplementation with vitamin A enhances peritoneal adhesion formation. Because vitamin E (VE) antagonizes the effects of vitamin A in certain systems (e.g., healing wounds) we hypothesized that supplemental vitamin E would prevent peritoneal adhesion formation in mice. One hundred and twenty-four adult female Swiss white mice were divided into two groups: 1) Mice fed a standard chow containing twice the NRC recommended daily allowance for mice, 2) mice fed the same chow supplemented with 300 mg of VE (DL-alpha-tocopherol) per kg diet. Mice were placed on their respective diets for three days prior to the surgical procedure and maintained on them throught the postoperative period. All mice ate and drank water ad libitum. The operative procedure consisted of a midline abdominal incision, under ether anesthesia, pinching of the parietal peritoneum with a fine hemostat and placing a 3-0 silk tie around the pinched peritoneal fold, according to the technique described by Ellis. The abdominal incision was closed with 3-0 nylon. On the 10th postoperative day, all animals were killed with ether and the peritoneal cavity was examined by two independent investigators under a blind code. There were no operative deaths. The presence and extent of adhesion formation was assessed and graded taking into account the number and strength of adhesions and the number of organs involved (Grade 0-Grade 4). There was a statistically significant decrease in the incidence (97% in control, 58% in the vitamin E-supplemented group, $p < 0.001$) and degree ($p < 0.01$) of peritoneal adhesion formation in the vitamin E-supplemented group of mice.

In conclusion, our data demonstrate that dietary supplementation with non-toxic doses of vitamin E is effective in lessening peritoneal adhesion formation.

C. Effect of Supplemental Vitamin E on Liver Collagen Content in Cirrhotic Rats Pre-and Post-Partial Hepatectomy

Vitamin E affects wound healing including fibroplasia and collagen accumulation. In this study, we examined the possibility that vitamin E may affect collagen accumulation in the cirrhotic rat liver, using as our test system CCl_4-induced cirrhosis. We previously showed that liver hydroxyproline concentration in such cirrhotic rats was twice that of normal rats. Thirty adult male S-D rats received 14 i.p. injections of CCl_4 (0.15 ml; 1:7 dilution) to induce cirrhosis. Ten days

later the rats were divided into two groups: Group A was fed a standard rat chow and Group B was fed the chow supplemented with 200 mg of vitamin E (DL-alpha-tocopherol cetate)/kg diet. Rats ingested the diet and water ad libitum throughout the experiment. Ten days later following start of the above diets, all rats underwent left hepatic lobectomy under pentobarbital anesthesia. The resected liver was analyzed for protein, fat, and hydroxyproline. On the third post-op. day, all rats were killed with ether, the remaining liver was removed and analyzed as above. Following partial hepatectomy, there was a significant ($p < 0.01$) increase in fat content 80 mg fat/g wet liver (pre-op.) to 113 mg fat/g wet liver (post-op.), and a significant ($p < 0.001$) increase in hydroxyproline content, 0.40 /mg protein (pre-op.) to 1.16 /mg protein (post-op.), in the group of rats fed a standard chow. No such increase in fat (83 mg fat/g wet liver to 87 mg fat/g wet liver) or hydroxyproline (0.35 /mg protein to 0.41 /mg protein) contents following partial hepatectomy was seen in rats fed the vitamin E-supplemented diet. A significant and similar decrease in liver protein concentration was seen in both groups post-hepatectomy. No significant differences in the weights of the resected livers or the regenerating liver remnants were seen between the two groups.

In conclusion, our data suggest that although dietary supplementation with vitamin E in non-toxic doses does not affect the liver protein, fat, and collagen content of rats with established cirrhosis, it prevents the increase in liver fat and collagen accumulation which follows additional injury (partial liver resection) and thus has a protective effect on the regenerating liver.

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D. Studies of Effects of Dietary Vitamin E Supplementation Subsequent to 1 November 1983 Progress Report

a. Protective Effect of Vitamin E in Rats with Acute Liver Injury

We have previously shown that supplemental vitamin E has a cytoprotective effect in the liver of rats with chronic CCl_4-induced liver cirrhosis. In this study, we hypothesized that vitamin E would have a protective effect in acute liver injury induced by D-Galactosamine. D-Galactosamine-induced injury has been thought to be due to a synergistic direct toxic effect and presence of intestinal bacteria and/or endotoxins. G-Galactosamine was used to induce acute "hepatitis" (1.5-2.0 g/kg body weight, i.p.). Rats were placed on either a standard commercial laboratory chow which contains several times more vitamin E than the NRC RDA for normal rats or the same chow supplemented with vitamin E (300 mg DL-alpha-tocopherol/kg diet) and 6 days later were given D-Galactosamine. There was significantly improved early (5-day) survival and late (14-day) survival in the vitamin E-supplemented group (Tables 7 and 8). The vitamin E beneficial effect was manifested also by decreased liver fat and collagen content and decreased SGPT level (Table 9). Because bacterial endotoxins have been implicated as playing a role in the pathogenesis of D-galactosamine hepatitis, the same experiment was carried out using germfree and conventional rats. There was significantly improved survival in both the germfree and conventional vitamin E-supplemented groups both at 5 and 14 days (Tables 7 and 8). There was no significant difference between conventional and germfree rats with or without vitamin E supplementation.

In summary (a) vitamin E improves the early fat and collagen accumulation in the liver, decreases SGPT level, and improves survival in the D-galactosamine experimental model of acute liver injury in both conventional and germfree rats; and (b) D-galactosamine toxicity is probebly not mediated through intestinal bacteria and/or endotoxins.

Among the biochemical changes induced by the administration of D-galactosamine include alterations in plasma lipid concentrations (1). Black et al. (2) demonstrated a defect in chylomicron catabolism and decreased lipoprotein lipase and hepatic lipase activity. Other investigators demonstrated hepatocyte mitochondrial damage, using both in vivo and in vitro systems, following D-galactosamine administration, resulting in uncoupling of the oxidative phosphorylation system (3, 4). Other studies suggested that the primary target of D-galactosamine is the cell membrane (5, 6). Farber and El-Mofty (5) demonstrated that D-galactosamine induces cell death by injuring the cell membrane and causing an influx of Ca^{2+} into the cell. Similarly, Schanne et al. (6) showed, using an in vitro hepatocyte culture system, that D-galactosamine toxicity was enhanced by increasing the Ca^{2+} concentration in the medium. Chlorpromazine, which inhibits Ca^{2+} influx into the cell had a protective effect in this system.

We have previously shown that dietary supplementation with vitamin E decreases CCl_4-induced liver toxicity in normal rats as manifested by a decrease in liver fat and collagen contents (7, 8). Following CCl_4 administration, there is an increase in liver fat and collagen content in rats. Following 70% partial hepatectomy in normal rats there is an increase in the regenerating liver fat and collagen content; similarly, 70% partial hepatectomy in cirrhotic rats results in further increase in regenerating liver fat and collagen content which can be prevented by dietary supplementation with vitamin E for 5 days prior to partial hepatectomy (7, 8). The observed increase in liver fat and collagen content following partial hepatectomy, CCl_4 administration and D-galactosamine administration can be seen as early as 24 hr following "injury" and it appears to be a sensitive biochemical manifestation of liver injury. Animals with elevated liver collagen and fat contents also had elevated SGPT levels following D-galactosamine administration. Dietary supplementation with vitamin E resulted in a decrease in liver collagen and fat contents and a concomitant decrease in SGPT levels. It would thus appear that dietary supplementation with vitamin E has a beneficial effect in rats with D-galactosamine hepatitis. Histologically, no differences between the standard chow and vitamin E-supplemented rats groups were seen. However, histologic examination was carried out only at 24 hr following D-galactosamine administration. Other investigators (9)

have shown that by 2 to 3 weeks following D-galactosamine administration, the liver of surviving rats shows nearly normal architecture. Yoshikawa and Kondo (10) demonstrated decreased serum vitamin E levels in patients with acute viral hepatitis. The same investigators also showe that vitamin E-deficient rats developed more severe liver enzyme abnormalities than vitamin E-supplemented rats.

Vitamin E has been shown to prevent oxidation of unsaturated fatty acids in biological membranes. This effect of vitamin E can result in both cell and mitochondrial "membrane stabilization". Quitanilla et al. (11) showed that vitamin E deficiency renders mitochondria more sensitive to lipid peroxidation, and Reddy et al. (12) demonstrated in liver microsomes that the protective effect of glutathione against thiobarbituric acid reactive product-toxicity can only be seen in the presence of vitamin E. These experimental studies suggest that vitamin E has a membrane stabilizing effect which could explain the effectiveness of vitamin E we have observed in the treatment of D-galactosamine toxicity. Hydrocortisone has also been shown to have a partial protective effect when given to rats with D-galactosamine hepatitis (13); this was believed to be due to its "membrane stabilizing" action. However, a free radical scavenger effect of vitamin E cannot be ruled out as a possible mechanism for its protective effect although there is no evidence to date to suggest that D-galactosamine induces cell injury through a free radical mechanism.

(continued next page)

TABLE 7

Early (5 day) survival of conventional and germ-free rats with and without dietary supplementation with vitamin E

Experimental group	No. of rats	Survivors/total no. (%) Standard diet	Vitamin E	p
S-D, conventional (1.5 g D-galactosamine/ Kg BW)	24	6/12 (50)	9/12 (75)	< NS
S-D, conventional (2 g D-galactosamine/ Kg BW)	26	3/13 (23)	8/13 (62)	< 0.02
Lewis, conventional (2 g D-galactosamine/ Kg BW)	19	2/10 (20)	5/9 (56)	< 0.02
Fisher, conventional (2 g D-galactosamine/ Kg BW)	16	2/8 (25)	6/8 (75)	< 0.05
Fisher, germ free (2 g D-galactosamine/ Kg BW)	22	2/11 (18)	5/11 (45)	< 0.05

TABLE 8

Late (2 week) survival of conventional and germ-free rats with and without dietary vitamin E supplementation

Experimental group	No. of rats	Survivors/total no. (%) Standard diet	Vitamin E	p
S-D, conventional (1.5 g D-galactosamine/ Kg BW)	24	5/12 (42)	6/12 (50)	< NS
S-D, conventional (2 g D-galactosamine/ Kg BW)	26	2/13 (15)	8/13 (62)	< 0.02
Lewis, conventional (2 g D-galactosamine/ Kg BW)	19	2/10 (20)	5/9 (56)	< 0.05
Fisher, conventional (2 g D-galactosamine/ Kg BW)	16	1/8 (12)	4/8 (50)	< 0.05
Fisher, germ free (2 g D-galactosamine/ Kg BW)	22	2/11 (18)	5/11 (45)	< 0.05

TABLE 9

Effect of dietary supplementation with vitamin E on liver hydroxyproline and fat contents[a]

Experimental group	No. of rats	Hydroxyproline /mg protein	Fat mg/g wet liver
Standard diet	15	..93 + 0.08	88 + 3.5
Vitamin E diet	15	0.12 + 0.02[b]	14 + 1.0[b]

[a] Results are expressed as mean values ± SEM.

[b] $p < 0.01$ (control *vs* vitamin E diet groups).

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Protective Effect of Vitamin E in Rats with Acute Liver Injury

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b. Beneficial Effect of Supplemental Vitamin E Following 90% Hepatectomy

In view of our previous studies suggesting that supplemental vitamin E has a beneficial effect in rats with D(+)galactosamine-induced liver injury, we examined the effect of vitamin E on survival in rats undergoing 90% partial hepatectomy. Adult male Sprague-Dawley rats (n=40) were placed either on a standard diet (65 IU vitamin E/kg diet) which supports normal growth, reproduction, and longevity of normal rats or a vitamin E-supplemented diet (300 mg DL-alpha-tocopherol/kg diet). Six days later, all rats underwent 90% partial hepatectomy under ether anesthesia. Rats were maintained on their respective diets and 5% dextrose drinking water ad libitum post-operative, and blood glucose levels were monitored. Animal survival was followed. All deaths occurred within the first 72 hr postoperatively. Vitamin E-supplemented rats had significantly increased early (24 hr) and late (14 days) survival when compared to control diet rats ($p < 0.01$ in each case, Table 10). Vitamin E-supplemented rats had significantly higher blood glucose values 12 hr postoperatively than control rats ($p < 0.02$, Table 11). At 48 hr postoperatively, serum glutamic pyruvic transminase levels were significantly ($p < 0.02$) lower in the vitamin E-supplemented group. Two weeks postoperatively, there was much less fat and collagen accumulation in the liver remnants of surviving vitamin E-supplemented rats when compared to that of control diet survivors ($p < 0.01$ in each case, Table 12). We conclude that supplemental vitamin E has a striking beneficial effect in rats undergoing major liver resection.

The mechanism for this beneficial vitamin E effect is not known. Zieve et al. (1) demonstrated 90% survival, complete liver regeneration and decreased liver steatosis following 90% partial hepatectomy in rats by simply adding 20% glucose to the drinking water. In this study we demosntrated that vitamin E-supplemented rats had higher blood glucose values in the immediate postoperative period; however, we have no evidence to suggest that vitamin E has a direct hyperglycemic effect. Animal survival in our control group was 20%; this is higher than the 0-10% figure usually reported (2,3) and is probably due to the use of 5% dextrose water in the immediate postoperative period. Gavino et al. (4) showed increased rates of liver regeneration following 70% partial hepatectomy in rats fed Santoquin, a synthetic anti-oxidant; no effect was seen in such rats fed the recommended daily allowance of vitamin E or a vitamin E-deficient diet. However, vitamin E-deficient rats had increased rate of liver peroxidation, whereas Santoquin-treated rats had diminished peroxidation. Vitamin E has been shown to protect cell and organelle membranes from lipid peroxidation, thus "stabilizing" the membrane and protecting organelle function. This may account for the decreased liver steatosis observed in this and other studies and the improved animal survival in the vitamin E-supplemented rats. Vic et al. (3) demonstrated 80% survival and decreased liver steatosis in rats undergoing 90% partial hepatectomy following a single injection of testosterone; they speculated that prevention of the accumulation of lipids in the hepatocytes by lysosomal degradation accounted for the decreased steatosis and improved survival.

Sekas and Cook (5) demonstrated increased SGPT levels following 70% partial hepatectomy and postulated that this was partly due to early release of enzymes from the cytosol of damaged hepatocytes. In this study, we observed elevated SGPT levels 2 days after 90% partial hepatectomy with lower values in the supplemented group.

Vitamin E may play a role in regulating the metabolic changes seen following major liver resection. Following 70% partial hepatectomy, major metabolic changes occur (6). Iris et al. (7) demonstrated impaired glucose tolerance 24 hr following 70% partial hepatectomy. Under similar conditions, Strecker et al. (8) showed a fall in serum glucose level and a rise in free fatty acid concentration. Nakatani et al. (9) demonstrated that following 70% partial hepatectomy, liver metabolism switches to predominant utilization of fatty acids as an energy source. Enhanced fatty acid oxidation leads to a reduced mitochondrial redox potential which in turn inhibits pyruvate dehydrogenase and possibly other Krebs cycle intermediates; this could account for the impaired glucose tolerance in these animals. Witting (10) demonstrated that increased levels of unsaturated lipids in the diet, causing an increased level of polyunsaturated fatty acids, increases vitamin E requirement in rats.

In summary, we demonstrated a significant beneficial effect of supplemental vitamin E, in non-toxic amounts, in rats undergoing 90% partial hepatectomy This effect was manifested as improved animal survival, partial prevention of early postoperative hypoglycemia, reduced SGPT levels 2 days postoperatively and reduced accumulation of liver collagen and fat content 2 weeks postoperatively. The mechanism of this beneficial effect is not known. It is possible that following liver injury, there is increased requirement for the powerful antioxidant effect of vitamin E in membranes which can provide "cytoprotection". However, in addition to its antioxidant and free radical scavenger effects, vitamin E could act as a regulatory agent in liver metabolism.

Table 10

EARLY (24 HR) AND LATE (2 WK) SURVIVAL OF RATS UNDERGOING 90% PARTIAL HEPATECTOMY WITH AND WITHOUT SUPPLEMENTAL VITAMIN E

	Survivors/Total		% Survival	
	Early	Late	Early	Late
Control	4/20	4/20	20	20
Vitamin E[α]	14/19*	13/19*	73	68

[α] There was one anesthetic death in the vitamin E-supplemented group.

* $P < 0.01$ (control vs. vitamin E).

Table 11

BLOOD GLUCOSE LEVELS IN RATS WITH AND WITHOUT SUPPLEMENTAL VITAMIN E FOLLOWING 90% PARTIAL HEPATECTOMY

	Blood glucose[α] (mg/100 ml)		
	12 hr	24 hr	48 hr
Control	39 ± 3	55 ± 4	62 ± 4
Vitamin E	56 ± 4*	61 ± 5	70 ± 6

[α] Mean ± S.E.M., n = 8-12.

* $P < 0.02$ (control vs. vitamin E).

Table 12

LIVER COLLAGEN AND FAT CONTENTS IN RATS WITH AND WITHOUT VITAMIN E SUPPLEMENTATION FOLLOWING PARTIAL HEPATECTOMY

	Hydroxyproline[α] (µg/mg protein)	Fat[α] (mg/g wet liver weight)
Control (n = 4)	0.85 ± 0.05	65 ± 3.8
Vitamin E (n = 13)	0.26 ± 0.02*	19 ± 1.6*

[α] Mean ± S.E.M.

* $P < 0.01$ (control vs. vitamin E).

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Beneficial Effect of Supplemental Vitamin E Following 90% Hepatectomy

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V Debridement of Burns: Surgical and Chemical

A. Surgical Debridement

The Use of the CO_2 Laser and a Hemostatic Scalpel for Burn Debridement

We have published our studies of the excision of burns with the CO_2 laser and a new heated scalpel. We undertook these studies because sepsis is still the main cause of death in patients with extensive, deep burns. The skin irreparably damaged by burns is highly susceptible to infection, and the severely burned patient has lowered "defenses". Although local and systemic chemotherapy has lowered mortality for sepsis, the incidence of severe burn-wound infection is still high and morbidity still too prolonged. We believe, as do many others, that prompt removal of irrevocably damaged, burned skin followed by immediate grafting with viable compatible skin diminishes sepsis, lessens functional abnormalities, lessens disfigurement, speeds recovery, and decreases mortality of patients with extensive third-degree burns. The problem is doing this safely and quickly. Accurate diagnosis of depth of burn is important for surgical escharectomy, but we will not discuss this problem. Totally compatible skih is now limited to autologous of syngeneic skin; how to extend the sources of skin in a way that does not require immunosuppression in the graft recipient is a problem yet to be solved. The use of artificial skin substitutes is under investigation by others.

Now as to methods for early surgical excision of the eschar: the conventional cold surgical scalpel is excellent where its use is feasible, but when the deep burn is extensive such excision often is difficult, involving prolonged anesthesia and considerable loss and replacement of blood. Burke, for example, has stated that "you need a big blood bank" to perform such excisions. If the operative procedures could be carried out with markedly less loss of blood and without significant local or systemic adverse effects, there would be considerable benefit to patients. The carbon dioxide laser, which we introduced, offers great advantages for excision of large, dep burns in term of blood loss, as shown by us (Stellar, S. et al.,Lancet 1:1945, (1971)); Levine, N., Ger, R., et al., Ann. Surg. 179:246(1974)); by Fidler and his colleagues (Fidler, J.P., Law, E., Rockwell, R.J., et al., J. Surg. Res.17: 1 (1974)); and Levine, N., et al., (Levine, N.S., Salisbury, R.E., et al., J. Trauma 15: 800(1975)). However, excision with current models is relatively slow and awkward. There are drawbacks, also, to the use of radiofrequency current electrosurgical units introduced for burn excision by Lewis and Quimby (Lewis, R.J., Quimby, W.C., Arch. Surg. 110:191 (1975)), that is, blood loss seems to be greater than when the carbondioxide laser is used and risks are associated with the electrical currents generated in the body by such units.

We were the first to test a new heated scalpel for its effects on wound healing and the excision of full-thickness burns (Arch. Surg. 117:13 (1982)).

The disposable blades of the heated scalpel system resemble conventional scalpel blades, except that their edges can be heated and the temperature controlled within narrow limits. The control mechanism compensates "instantaneously" for varying lsoses of heat depending on the vascularity of the tissues and rate of cutting. Cutting is done by the blade's sharp edge and hemostasis results from direct transfer of heat; no electric currents are generated in the tissues. We compared the healing of dorsal skin incisions performed with either the cold or the heated scalpel in male Sprague-Dawley rats anesthetized with pentobarbital. A preliminary series of experiments examining the breaking strengthf of incisions made with the hemostatic scalpel at the lowest (110°C) and highest setting (180°C) and intermediate temperatures by increments of 20°, showed similar values for all these temperatures at 7 days postoperatively. In another series of experiments, the hemostatic scalpel was set at 180°C to achieve essentially complete hemostasis while making the incisions with a simple rapid stroke.

The "cold and heated" incisions were closed with fine stainless steel sutures. The breaking strengths of both types of incisions were tested in the fresh state and after fixation with formaldehyde 7, 14, 21, 28, 35, and 42 days postoperatively. The only difference of statistical significance, p 0.05, was noted at 21 days postoperatively with the value of breaking strength slightly favoring the conventional scalpel. We also studied wound resistance to infection by inoculating skin incisions made in rats with either the heated or the cold scalpel, with up to 10^8 viable Pseudomonas aeruginosa or Staphylococcus aureus No wound infection developed in either group, and, in fact, all the incisions (heated or cold scalpel) contaminated with S. aureus showed a striking increase in breaking strengh. This finding is at variance with the result in incisions performed with the carbon dioxide laser or the electrosurgical unit, where contamination with S. aureus demonstrates diminished resistance to infection as compared to incisions performed with the cold scalpel (Madden, J., Edlich, R., Custer, J., et el., Am. J. Surg. 119: 222 (1979)).

We have shown that the heated scalpel allows excision of 3^o burns in pigs and humans with considerable less loss of blood than when the usual cold surgical scalpel is used. Skin grafts applied immediately after excisions with the heated scalpel had excellent rates of success, similar to those of grafts applied immediately after excisions with the cold scalpel.

Thus, there are a number of advantages of the heated scalpel:

1. In shape and sharpness it resembles the usual surgical scalpel.

2. The scalpel blades are disposable, and can be made in a variety of shapes and sizes.

3. The scalpel may be used heated or cold, depending on the surgical step required.

4. The control mechanism compensates for varying heat losses depending on the type of tissue incised and the rate of cutting. We think that a small amount of bleeding is desirable to avoid tissue damage caused by excessive heat. By depressing a switch in the handle the blade temperature immediately rises momentarily to 260°C for "sealing" larger vessels. Larger perforators may also be controlled using a radiofrequency electrosurgical unit in the coagulation mode.

5. The heated scalpel is easier to manipulate and use than the carbon dioxide laser, and excisions with it can proceed faster than with the laser.

6. The scalpel thermally transfers heat directly to the tissue and is electrically insulated from the patient. No electric current passes through the patient. As a consequence, a grounding pad is not needed and the risk of accidental radiofrequency electrical current burns at grounding sites can be eliminated. Also, the muscle stimulation associated with the passage of radiofrequency electrical current through the body is eliminated.

7. Blood loss is lessened considerably when excision of burned skin is carried out with the heated scalpel, with little damage to the underlying tissue as indicated by the excellent success rate of immediately applied skin grafts.

8. The heated scalpel is useful in other surgical operative procedures for the control of blood loss.

Clinically, the hemostatic scalpel has been used by others in many areas of general surgery and the surgical specialties, especially where large incisions or tissue transfers are involved. The modified radical mastectomy exemplifies the value of the method (Pilnik, S., Steichen, F.M.). We (Steichen, F., Levenson, S.M., Problems in General Surgery 2: 11017(1985)) emphasize that the degree of hemostasis obtained depends on the amount of heat delivered, that is, the total caloric input. The caloric input is a function of blade temperature, duration of blade-tissue contact, the contact area, and the type of tissue, especially its vascularity. In order to use the hemostatic scalpel successfully, the surgeon has to learn to use these variables to full advantage.

B. Chemical Debridement

a. Background

Another approach to the problem of removal of devitalized skin is the use of topically applied chemicals for debridement. Chemical debridement of burns offers the potential of minimizing blood loss and not requiring anesthesia or differentiation of depth of burn by the surgeon. This concept, and practice, is old, dating back to biblical times, but there is still no commercially available preparation which meets all the requirements of a suitable (ideal) chemical debriding agent for clinical use. We (Levenson, S.M.: J. Trauma 21:735(1981)) have pointed out that chemicals for debridement should have the following attributes: (1) actions that differentiate unburned skin, partial-thickness burns, and full-thickness burns, debriding only non-viable tissue and not injuring viable tissue; (2) rapid and controllable debridement; (3) no local or systemic toxicity (including metabolic, physiologic, and immunologic) or sensitization; (4) no predisposition to local or systemic infection; (5) no interference with the anti-microbial action of topical or systemic chemotherapeutic agents which conversly should not interfere with the debriding activity; (6) immediate or very prompt successful skin grafting after debridement should be possible; (7) compatibility with complemental (simultaneous or sequential) surgical excision. (8) Ideally, the agent should be a known compound; this would make it easier to characterize, synthesize, and control production and reproducibility.

Travase, an enzyme preparation (largely proteolytic) prepared from B. subtilis, and incorporated into a hydrophobic ointment base is the most widely used commercial preparation. Dimick has reported that Travase hastens the separation of burn eschar, shortening the period to initial grafting, and reducing hospital stay. In general, though, the action of Travase is slower (several days) than desirable, except when applied to dermal burns of the dorsum of the hands. In the latter case, Gant has reported that after about 24 hours, the devitalized tissue can be readily scraped away and skin grafts applied immediately take very well. Dimick has confirmed this. It appears to be non-injurious for unburned skin and no local or systemic toxicity has been reported. There is, however, an increase in fluid loss when Travase is applied early after the thermal injury; this has limited the amount of burn surface that can be treated at any one time. Travase does not attack devitalized fat and when Travase is used for debridement of full-thickness burns of patients, skin grafting cannot be done without first excising some remaining underlying devitalized tissue or allowing time for spontaneous sloughing and beginning granulation.

There is some evidence that sepsis may be increased by the use of Travase although in Dimick's series this did not seem to be the case. There has still not been, as far as we know, a prospective randomized concurrent clinical study with two groups of patients in which one group is treated with Travase, the other not.

b. Our Experiments

1. Methods

We used pigs as one of the species of experimental animals since evidence suggests that the burned skin of the pig may be closer to the burned skin of patients than that of rats, mice, guinea pigs, rabbits, and dogs, animals which have been used largely in earlier studies with chemical debriding agents. Dogs and rats were also used in our studies, especially for skin grafting experiments after debridement, since the subcutaneous tissue of pigs is relatively avascular. Standard contact and hot water burns of known depth and area, both partial thickness and full-thickness, were produced under pentobarbital anesthesia at times supplemented with ether.

In vitro tests with burned and unburned skin were carried out to assess the effect of the chemical agents and determine conditions of concentration, pH, synergisms and antagonisms. Pieces of burn eschar of fixed dimension and weight were incubated with the chemicals and enzymes under test, generally at 37°C in order to speed up the reactions for assay purposes. At varying times, the appearance of the test burn eschar was noted and the nitrogen and hydroxyproline contents of the media and the suspended, fragmented eschar determined as indices of the solubilization and fragmentation of the eschar. Unburned skin was tested similarly simultaneously to detect possible effects of uninjured skin.

Various chemical agents, enzymatic and non-enzymatic, were tested, singly, sequentially, and in combination in different media at controlled pH's and ionic strengths. Appropriate controls, e.g., inactivated enzymes, vehicles, etc. were studied simultaneously. These in vitro studies were complemented by in vivo studies to assess rates of debridement and possible local and systemic toxicity.

A standardized experimental model devised in our laboratory and reported previously was used for screening debriding activity in vivo. Under pentobarbital anesthesia, rows of circular full-thickness burns, each about 20 cm^2 and each about 5 cm from the next burn, were produced on each side of the clipped dorsal skin of female or castrated male white Hampshire-Landrace pigs weighing about 40-50 pounds. The contact burns were created by applying a copper cylindrical block of fixed dimensions at fixed temperatures for predermined times. In a series of experiments, the time and temperature required to produce the depth of burn desired was determined by sequential biopsies and following the course of healing and sloughing of the burned skin. A temperature of 83-85C for 75 seconds produces a full-thickness burn on the skin of the back of pigs of this size. Standardized partial-thickness burns were produced by shortening the time of contact. Similar burns were produced on dogs.

A number of different chemical agents, both enzymatic and non-enzymatic, were studied in the same pig; the sites used for specific agents were chosen at random. The various enzymes and chemicals were incorporated into various vehicles, e.g., ointment bases, in known concentrations, with and without chemotherapeutic agents.

In other experiments, hot water burns (100C, 6-10 seconds, depending on the size of the rat) were used to produce deep burns on the abdomen or back of rats, involving up to 20-25% body surface. Fixed amounts of the preparations were applied to the burned areas daily (or more often depending on the specific experiment) under a gauze dressing covered with plastic and then held in place by roller gauze, stents or plaster casts.

The rates of debridement of the full-thickness burns were determined and the character of the underlying base assessed grossly and by its ability to accept auto- or homo-full thickness skin grafts, immediately or at various times after debridement. The progression of the debridement, granulation tissue formation and skin grafting was followed by serial clinical, photographic and bacteriologic examinations, including quantitative bacterial counts in some experiments.

Effective debriding agents were also tested on unburned skin and partial thickness burns.

To determine possible local toxicity the following types of experiments were done: 1) Effect of the chemical agents with and without chemotherapeutic and antibiotic agents (and appropriate control applications) on reepithelialization of partial thickness burns; and 2) Effect on underlying tissue (a) appearance of the tissue, (b) fate of full-thickness skin grafts applied to the area immediately or later after the wound was allowed to granulate.

To assess possible systemic toxicity, 3^{o} burns were made, involving 15-40% of body surface of pigs and 20-25% body surface of rats. The rats received 0.9% sodium chloride, 10% of body weight, intraperitoneally right after burning and 5% glucose in saline by mouth ad libitum during the first day after burn. All the animals ate and drank ad libitum during the pre- and post-burn periods. The rats were fed a commercial rat chow and the pigs commercial pig and dog chows. The agents were applied by the techniques established as most effective by the experiments already described. Full-thickness burns were used; contact for the pigs, produced by repeated contiguous applications of a rectangular copper block 83C for 75 seconds and hot water burns on the rats (100C, 6-10 seconds depending on size of rat). Control animals received similar burns but were treated with the vehicle or inactivated enzyme preparations for the same length of time as the experimental animals; their burn eschars were then excised and thereafter both groups were handled identically. Silver sulfadiazine ointment was applied immediately after debridement and excision under gauze dressings, changed 2 days later and reapplied for 3 more days. Thereafter, for pigs the areas were left exposed. Body weight, hematocrit, plasma total solids, plasma total protein and albumin, blood sugar, blood urea nitrogen, plasma sodium, potassium, chloride, calcium, phosphate, plasma creatinine, CPK, LDH, SGOT, SGPT and alkaline phosphatase were measured serially for many weeks; the pig has little if any plasma bilirubin or uric acid. The activity and behavior of the animals were noted.

2. Studies with Bromelain

Our initial observations showed that Bromelain, an enzymatic preparation derived from pineapple stems with proteolytic and mucolytic activity was more effective in vitro in "solubilizing" third degree burn eschar than any other enzymatic preparation we tested, that very rapid debridement (hours) of third degree contact burns in pigs can be accomplished with a single application of 50% Bromelain (buffered to pH 7.4). The healing of partial thickness burns of pigs was not slowed by the Bromelain.

Experiments were then conducted to follows up our initial studies in order to devise methods for the safe, rapid chemical debridement of deep burns.

A. Pigs. When commercial Bromelain 1200 (Dole Co., Honolulu, Hawaii) or a further purified preparation of Bromelain (Miles Laboratories, Elkhart, Indiana) was incorporated in a vanishing cream (Pond's Oily Skin Treatment, Cheeseborough Ponds Inc., New York, NY) buffered to pH 7.4 with Tris-buffer or in Tris-buffer, the rate of debridement was proportional to the concentration of Bromelain used.

A single application of a 50% concentration of Bromelain to the intact burned area resulted in an obvious debriding effect in 2-4 hours and complete debridement in 12-24 hours. The debriding action by 12-24 hours consisted of some solubilization of the burn eschar and a partially lytic effect at the interface between the burn eschar and the underlying viable tissue so that the eschar could be really dissected off with a throat stick with minimal, and often no, bleeding. The underlying tissue looked undamaged by the Bromelain. At times there were occasional thrombosed vessels in the underlying subcutaneous layer.

When the time of Bromelain was shortened to about 8 hours, the degree of solubilization was less, but dissection with a throat stick could be carried out with relative ease.

There was little apparent difference in the debriding action of the various

preparations of Bromelain, and the results were similar whether the Bromelain was incorporated in the buffered vanishing cream or in the Tris-buffer alone.

Bromelain heated in the Tris-buffer to about 100C for 3.5 hours lost its debriding action.

The Tris-buffer by itself and the vanishing cream alone hydrated the burn eschar but did not solubilize it when applied forthe same times and in the same ways as when the active Bromelain was used. It is possible to separate the hydrated burn eschar, not acted upon by Bromelain,from the underlying viable tissue by the incision of the eschar and thrusting a throat stick beneath it. However, considerably more force is required than when Bromelain is used. The difference was readily distinguishable when the surgeon did not know before hand which burn eschar had been treated with Bromelain and which had not, e.g., when the period of application was less than 8 hours. For longer applications, the surgeon could tell which eschar had been treated with Bromelain because of the obvious beginning of the solubilization, but again there was no question about the dissection being very much easier after Bromelain.

When Bromelain was used in a concentration of 20%, debridement was accomplished in 24-48 hours; concentrations of 10% and 5% Bromelain had progressively slower debriding activity, the latter requiring days.

B. Rats. When Bromelain in a concentration of 50% in buffered vanishing cream or Tris-buffer was applied to hot-water burns of Sprague-Dawley rats the rates of debridement varied depending on: 1) the age and weight of the rat, and thus the thickness and character of the skin; and 2) the location of the burn. The younger the rats, the faster and the more effective the Bromelain. In fact, when the burns were on the backs of the rats, debridement occurred in 4-6 hours in rats weighing 100-150 g (5-7 weeks of age), while little or no debridement occurred in 8-10 hours in rats weighing 300-400 g (3-4 months of age). In these experiments the Bromelain was applied for 2-3 hours, then washed off and Bromelain reapplied for another 2-3 hours, etc. In contrast to back burns, when the abdominal skin was burned, debridement was very rapid in the young rats (2-3 hours) and occurred in the older rats in 3-4 hours. As in the case of the pigs, the action of Bromelain consisted of some solubilization of the burn eschar and partial lysis at the interface between the burn eschar and the underlying viable panniculus carnosus so that the eschar could be separated readily by blunt dissection or wiping, the latter requiring proportionately somewhat less time than major solubilization of the eschar.

After debridement of the burn eschar, the underlying tissue appeared healthy. Skin grafts, full-thickness homografts from other Sprague-Dawley rats or partial thickness xenografts from pigs, became vascularized (proven by injections of colloidal carbon and gross and microscopic examination) and were then rejected. The behavior of the grafts was similar to that of xeno- or homografts applied after surgical excision of the burn eschar.

When inbred Fischer rats were used, the skin allografts took very well and persisted indefinitely, just as occurred when the burn eschars of paired rats were excised surgically and allografted.

C. Dogs. Bromelain, 50% in buffered vanishing cream or Tris-buffer, was effective in debriding third degree contact burns of dogs when applied as a single application and left in place for 18-24 hours. Again, the debridement was characterized by some solubilization of the burn eschar and modification of the interface between the burn eschar and the underlying viable tissue so that removal of 90-100% of the remaining eschar by blunt dissection or wiping was readily accomplished. The base looked healthy, and autogenous skin grafts applied immediately took if performed under strict asepsis.

If the burn eschar is cross-hatched with a scalpel down to, but not into the underlying viable tissue, the rate of debridement is hastened.

Effect of Silver Sulfadiazine on Debriding Activity of Bromelain

When Bromelain is mixed with a current commercial preparation of silver sulfadiazine (Silvadene, The Marion Laboratories, Kansas City, Missouri), its debriding activity was slowed moderately, about 10-20%, when tested in rats and pigs with third degree burns, hot water and contact respectively.

Effect of Bromelain on Healing of Partial Thickness Burns and on Uninjured Skin

Bromelain applied to partial thickness burns of pigs in the concentrations and for the times required for the very rapid debridement of third degree burns does not interfere with epithelization of the partial thickness burns and does not appear to injure unburned skin of pigs, dogs and rats. In fact, 50% Bromelain in Tris-buffer may be applied for as long as 24 hours to partial thickness burns of pigs without slowing re-epithelization.

If 50% Bromelain is applied to unburned skin which has been cut during shaving, irritation and some injury occurs at the cut sites.

Bromelain Debridement of Large Third Degree Burns in Rats and Pigs

A. Rats. Experiments to detect possible systemic toxicity of Bromelain when applied to moderately large third degree burns were conducted with Sprague-Dawley male rats. We found that 50% Bromelain in Tris-buffer or buffered vanishing cream applied promptly after third degree hot water burns involving 20-25% of the skin area of rats (weighing 150-200 g) results in prompt debridement (3-4 hours). Refrigerator stored pig-skin applied to the debrided area became vascularized (proved by i.v. injection of colloidal carbon and gross and microscopic observation) and then undergoes typical "first-set" rejection at 7-10 days. We used pigskin rather than autogenous skin for these experiments because we did not want to create another wound in these rats. Only 1 of 25 rats so treated died, on about the 10th day. Autopsy revealed some clear peritoneal fluid, no petechiae, and no obvious cause of death.

In other experiments, the application of Bromelain was delayed for 24 hours after burning and homografts from other Sprague-Dawley rats rather than xenografts were used. The time for debriding was little different from the preceding experiment. The homografts took well, vascularization was demonstrated by the i.v. injection of colloidal carbon, and the rats survived.

In a third series of experiments, rats were burned over 20% of their body surface as above. Twenty-four hours later, they were randomly divided into two groups. One group was treated with 50% Bromelain in buffered vanishing cream, the other with the buffered vanishing cream. Several hours later, when debridement by Bromelain was accomplished, the burn eschar of the group treated with vanishing cream was excised, attempting to stay above the panniculus carnosus, since that was the plane of separation of the burn eschar by the Bromelain debridement. Reconstituted lyophilized pig skin was applied to the open wounds of both groups of rats.

There were no differences in behavior, activity, weight gain, hematocrit, total solids and plasma CPK levels of the two groups of rats, which rose slightly after burn, in contrast to our findings with pigs.

B. Pigs. In our initial experiment, six pigs were burned over 30-40% of their body surface. Rapid debridement was accomplished (30-50%) and the wounds covered with lyophilized pig skin, ointment and dressed for 5 days, the dressings being changed daily thereafter, the wounds were left exposed to the air.

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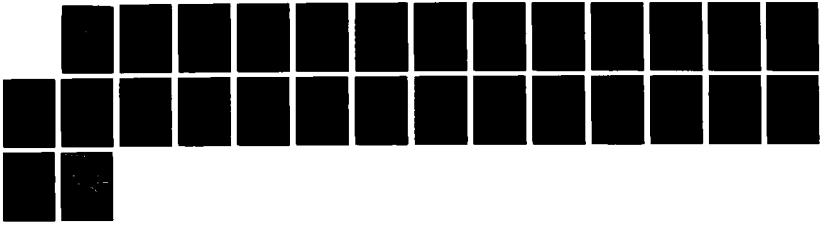

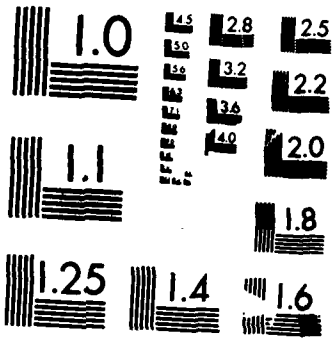

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vanishing cream were studied concurrently. The pigs continued to eat, drink and to grow. The debrided areas formed healthy granulation tissue, and there were no blood chemical abnormalities attributable to the chemical debridement. Following the burn itself, and unmodified by the chemical debridement, was a very prompt rise in plasma LDH and CPK. The rises in plasma LDH and CPK occurred within hours after burning in the pigs and then fell towards normal in about 5-7 days. Plasma SGPT, SGOT and alkaline phosphatase were relatively unchanged.

In another series of experiments, young actively growing pigs were divided into two paired groups, each pair being matched according to their body weight and pattern of weight gain. All pigs received third degree contact burns on both flanks, involving 15-20% of body surface. During the pre-burn period, the pigs were housed together, 10-12 in each of two large pens. After the burn injury, the pigs in one experiment were housed together for 7 days and then individually; in another experiment the pigs were housed separately throughout the post-burn period.

After burning, the burned areas of all pigs were left exposed to the air for one day. The burns of one group of pigs in each experiment were then treated in our usual way with 50% Bromelain in buffered vanishing cream, while the other group was treated with the buffered vanishing cream. Eighteen to 24 hours later, the dressings were removed. The areas treated with Bromelain had been partially fragmented and the remaining eschar was removed readily by blunt dissection or wiping. The underlying viable tissue appeared healthy. The areas treated with the vanishing cream alone were intact and were then excised by sharp scalpel dissection. The denuded areas on all pigs were then covered with silver sulfadiazine ointment and a dressing applied. The dressings were changed two days and five days later, following which neither ointments nor dressings were applied. The wounds remained clean. Scabs developed within a short time following exposure of the burn and healthy granulations formed. There was gradual contraction of the wounds, but no epithelization except from the periphery since the burns had destroyed the full-thickness of the skin.

There were no significant differences among the pigs debrided with Bromelain and those in which the burns were treated with buffered vanishing cream and then excised surgically in behavior, activity, apparent food intake (food intake was judged grossly, but not measured), weight gain, hematocrit, plasma total solids, plasma total protein, plasma albumin, sugar, urea nitrogen, sodium, potassium, chloride, calcium, phosphate, creatinine, CPK, LDH, SGOT, SGPT and alkaline phosphatase. In both groups, there were prompt rises in plasma CPK, LDH, SGOT and SGPT within 24 hours after burn, before the application of Bromelain or the vanishing cream alone. These rises were especially high (a factor of about 5) for CPK and SGOT. The plasma CPK returned towards normal by the 10th day and there was then a secondary rise 3 weeks following burns. A similar pattern was seen for the plasma SGOT but the secondary rise was much less. Plasma LDH also rose within 24 hours (a factor of 2) and then fell gradually to normal by the 10th post-burn day. The rise in plasma SGPT was slightly slower as was its fall, normal levels being reached by day 20. The changes in the plasma enzymes were no different in those pigs subjected to chemical debridement with Bromelain than in those pigs treated with vanishing cream and surgical excision.

Control unburned pigs, 2 in number, showed no plasma chemical changes.

Sterilization of Bromelain

Sterilization of Bromelain solutions was accomplished without interfering with its debriding activity. For this, Bromelain was added to the Tris-buffer, pH 7.4, stirred gently for 10 minutes, and then centrifuged for 10 minutes at 2000 rpm. The precipitate was discarded. The supernatant was divided into two equal parts, one of which was not treated further while the other was sterilized by filtering it through a 0.22 μ Millipore membrane. Both solutions showed equal debriding activity. Heating powdered Bromelain for 1-3 hours at 160°C for sterilization destroyed its debriding activity.

Behavior of Current Commercial Bromelain Subjected to Elution from Sephadex G-100 and DEAE Ion Exchange Columns

The currently available commercial Bromelain was partially solubilized in Tris-buffer, pH 7.4 at room temperature (about 22-23 C) so that the nitrogen content of the solubilized fraction was 250-350 mg N/10 ml of buffer. Aliquots of such solutions were introduced in amounts containing about 175 mg N into a Sephadex G-100 column (36 in. long and 2.6 cm in diameter) and eluted with Tris-buffer, pH 7.4, or in amounts of 1300 to 2600 mg N into DEAE ion exchange columns containing 120 and 200 g of the dry resin respectively in columns 38 cm long and 5 cm in diameter and 52 cm long and 5 cm in diameter respectively and eluted with 0.02 M and 0.5 M sodium citrate pH 6.0. The eluates were collected in arbitrary-fractions in each case.

In each case, all the added Bromelain nitrogen was recovered. The majority of the nitrogen eluted from Sephadex G-100 column was in fractions 5-11 and accounted for most of the Bromelain nitrogen which had been introduced into the column. In each fraction, the proteolytic activity against casein and the ability to attack burn eschar in vitro and in vivo (rats) and nitrogen content of the eluate correlated closely. A similar pattern was noted for the DEAE eluates. The bulk of the Bromelain nitrogen introduced into the DEAE columns was recovered in one experiment in fractions 2, 3 and 4 (500 ml eluate); fraction 1, 300 ml contained very little nitrogen. In another experiment, the bulk of the Bromelain nitrogen was recovered in fractions 5-10 (100 ml eluate; fractions 1-4 in this run contained 700 ml eluate). These two experiments differed in the amounts of Bromelain solution introduced into the columns and the sizes of the columns; the details about these factors have already been mentioned. In each case, there was a high correlation among the nitrogen content of an eluate fraction, its ability to digest casein in vitro, and its ability to attach burn eschar in vitro and debride burn eschar in vivo (rats).

When the various active fractions were "equalized" on the basis of nitrogen content, their proteolytic activities against casein were similar.

The rates of casein digestion relative to different concentrations of nitrogen in the various elutions showed that in each case, the action against burned skin in vitro was considerably greater than against unburned skin: there was little evidence of any fraction attacking the unburned skin. A similar situation was seen in vivo with burned rats.

Neither the commercial Bromelain nor any of the eluate fractions showed lipase activity. On the other hand, the commercial Bromelain and certain of the eluates showed amylase activity; for the DEAE and Sephadex eluates, amylase activity was greatest in those fractions which contained the most nitrogen, had the greatest proteolytic activity against casein, and the greatest debriding activity against burned skin. There was some amylase activity, however, though less, in other fractions.

3. Chemical (Non-Enzymatic Debridement)

Three groups of non-enzymatic chemical compounds were studied in vitro and their effects on burned and unburned skin noted: 1) mercaptans which may cleave certain links in proteins (disulfide, ester) and which will also cleave protein-carbohydrate linkage; 2) certain metal ions which cause a weakening of protein fibrillar structure, and 3) compounds which cause adisaggregation of mucopolysaccharides.

In the first category, four compounds were studied: a) penicillamine, b) N-acetyl cysteine, c) mercaptoethanol, and d) cysteine ethyl ester.

In the second category were salts of zinc, cobalt and manganese.

In the third category were: a) 8-OH quinoline 5 sulfonate, b) 8-OH quinoline, c) sodium salicylate, d) sodium pyrophosphate, e) sodium tripolyphosphate, f) sodium citrate, and g) sodium EDTA.

To test samples of burned and unburned skin were added to the test chemical solutions and incubated for 24 hours at 37 C. Cysteine ethyl ester (0.01-0.1M), 2-mercapto ethanol (0.5-1.0M), N-acetyl cysteine (0.005-0.01M) and penicillamine (0.25-0.5M) demonstrate the desired selective action on burned skin as opposed to unburned skin in vitro.

At the request of the Army Research and Development Command our investigations of the chemical debridement of burns was discontinued as part of our Army Research support when we received support for this phase of our research from the National Institutes of Health.

(continued next page)

VI Wound Healing Accelerating Effect of *Staphylococcus aureus*

We have carried out studies of the dramatic wound healing accelerating effect of *Staph. aureus*. We first made this observation serendipitiously when we were attempting to determine whether incisions made with a new heated hemostatic scalpel we were using are more susceptible to wound infection following purposeful bacterial contaminatnion of the incisions than incisions made with the usual cold surgical scalpel (Kan-Gruber, *et al.*, Surg. Forum 31: 232(1980)) and Surg. Forum 32: 76(1981)), as has been reported by others for incisions made with an electrosurgical unit and the CO_2 laser (Madden, *et al.*, Am. J. Surg. 119:222 (1979)).

In a typical experiment, dorsal skin incisions (7 cm) are made aseptically in anesthetized Sprague-Dawley male rats. In half of the rats, chosen at random, 0.2 ml of an 18 hour culture of bacteria of measured concentration is instilled into the wound along its length and applied along the surface of the wound within one hour after closure with interrupted fine stainless steel sutures. 0.2 ml sterile culture medium is used in a like fashion in the other half of the rats. (We had shown in other experiments that the instillation of this sterile culture medium did not affect wound healing). All eat our standard rat chow and drink tap water *ad libitum*. At various times postoperativley, the rats are killed by ether, the wounds cultured and the breaking strengths of the incisions measured in the fresh state and also after fixation in 10% formalin. The wounds are also prepared for histological examination.

We have reported in previous progress reports that the accelerating effect of *Staph. aureus* on the gain of wound strength was evident four days postoperativelv, the earliest time tested, was maximal at 7-10 days, and was still present at 28 days postoperatively, the longest time tested. The accelerating effect was seen when as few as 10^2 *Staph. aureus* were introduced into the wound and this effect increased progressively as the number of *Staph. aureus* introduced was increased to 10^8. The data indicated that both accumulation of reparative collager and its cross-linking were increased. There was no evidence of infection on gross examination in any of a very large number of experiments; on histological examinat'or only on occasional micro-abscess was seen in some rats.

The wound healing accelerating effect was seen also when incis'ons of germfree rats were inoculated with *Staph. aureus*, the rats being maintained thereafter in isolators using germfree husbandry techniques, an indication that no other v'ab'e microorganisms need be present in the host for the wound healing effect due to *Staph. aureus* to become evident. There was little difference in the wound healing accelerating effect whether the incisions were inoculated with the *Staph. aureus* immediately after closure or 24 hours later as judged by the breaking strengths on the seventh postoperative dav.

The wound healing acce'erating effect was not limited to a single strain of *Staph. aureus*: seven strains with varying enzvme characteristics demonstrated the marked accelerating effect. In sharp contrast, neither *Staph. epidermidis* (2 strains), *Staph. hominis* (1 strain) nor *Ps. aeruginosa* (2 strains) showed this effect. the *Staph. epidermidis* was tested both in conventional and germfree rats

when the *Staph. aureus* was injected at wounding there was a slight but statistically significant increase in wound breaking strength. The increase was much less than that which follows the inoculation of the wound with *Staph. aureus* When only one of two incisions in the same rat was purposely inoculated with the *Staph. aureus*, only the former showed the accelerated healing. Thus, the effect of *Staph. aureus* appears to be primarily local when the purposeful contamination is at the time of wounding. There may be a systemic component when germfree rats were noncontaminated by oral feeding of *Staph. aureus* two weeks before wounding the incisions gained strength faster than the germfree controls. In another experiments carried out with germfree rats noncontaminated with *Staphylococcus epidermidis* were negative

No wound healing accelerating effect was seen when a millipore filtrate of an actively growing culture of Staph. aureus was instilled into the incision at closure.

In a preliminary experiment, inbred Fischer male rats. 100-110 g. were anesthetized and burned on their backs 100°C water for 10 seconds (3° burn; 2.6x4.0 cm oval). Saline (0.85% NaCl) was injected i.p. in an amount equivalent to 5% of their body weight right after burning and the rats drank 5% glucose in saline ad libitum for the next 2 days and then tap water. They ate a standard commercial rat chow ad libitum throughtout the experiment. Twenty-four hours after burning, the burn was excised and covered with a full-thickness skin graft (syngeneic) of the abdominal skin of 250 g Fischer male rats. The grafts were stapled to the surrounding skin. Five to six small holes were cut into the grafts. Saline was applied along the entire perimeter of the grafts of the control rats as well as to the 5-6 holes cut into the skin grafts while $5x10^6$ Staph. aureus was similarly applied to the grafts experimental rats. In both groups of rats, the take of the grafts was 90-100%. At postoperative day seven the grafts of the control rats appeared less pink than those of the Staph. aureus treated rats. This will be pursued, applying the Staph. aureus in varying doses over the recipient bed immediately before the placement of the syngeneic skin graft. The rate of vascularization will be followed using a colloidal carbon injection technique with which we have had experience in our laboratory.

We have reviewed much of the relevant literature and discussed some of the mechanism(s) we consider likely as underlying the wound accelerating effect of Staph. aureus in previous Progress Reports and in our paper "Wound Healing Accelerated by Staphylococcus aureus", Levenson. S. M., Kan-Gruber, D., Gruber, C., et al., Arch. Surg., 118: 310-320 (1983); and will not reiterate that material here.

In considering possible mechanism(s) we asked the question "Do some of the key differences between Staphylococcus aureus and Staphylococcus epidermidis account for their different effects on wound healing? In addition to the presence of coagulase (free or bound) and α-toxin in almost all Staph. aureus subspecies, certain endonucleases and cell-wall components (ribitol and protein A) are present in Staph. aureus and not in Staph. epidermidis. Ratcliff, McCool and Catalona (Cell. Immunol. 57: 1 (1981)) found that Protein A greatly enhances the production of interferon by macrophages. It is well established that macrophages (especially activated macrophages) play an important role in wound healing. (Ross, R., in Wound Healing and Wound Infection: Theory and Surgical Practice, pp. 1-10 (1980)); (Leibovich, S. J., Ross, R., Am. J. Pathol. 84: 501 (1976)); (Clark, R. A., Stone, R. D., Leund, D. Y. K., et al., Surg. Forum 27: 16 (1976)); (Greenberg, G., Hunt, T. K., Cell Physiology, 26: 353 (1978)); (Thakral, K. K., Goodson, W. H., III, Hunt, T. K., J. Surg. Res. 26: 430 (1979)); (Hunt, T. K., Andrew, W. S., Halliday, B., et al., The Surgical Wound, pp. 1-18 (1981)); (Cassey, W. J., Peacock, E. W., Jr., Chvapil, M., Surg. Forum 27: 53 (1976)); (Diegelmann, R. F., Kaplan, A. M., McCoy, B. J., et al., Proceedings of the Eleventh International Congress of Biochemistry, Toronto, National Research Council of Canada, p. 502 (1979)); (Diegelmann, R. F., Cohen, I. K., Kaplan A. M., Plast. Reconstr. Surg. 68: 107 (1981)).

We have tested strains of Staph. aureus with varying protein A production to determine whether protein A production by Staph. aureus correlates with accelerated wound healing. The data showed that the wound healing accelerating effect of Staph. aureus ATCC #10834 which is very low in protein A and Staph. aureus ATCC #12598 which is high in protein A have similar wound accelerating effects when each is inoculated at high concentration ($3x10^9$) into dorsal skin incisions of rats. This suggests that protein A may not be a necessary factor for the wound healing accelerating effect. We plan to use protein A by itself in some experiments.

There are important differences between our findings with Staph. aureus and those of Enquist and his colleagues who found an accelerating effect on the gain of strength of paramedian incisions in rats when the wounds were contaminated with various species of gram negative bacteria. In their experiments, the maximal effect was a 50% increase, and this increase was not evident until about the 14th day. In fact, in their experiments, the control non-contaminated wounds were stronger at 7 days postoperatively than the purposefully contaminated wounds, whereas in our experiments the accelerating effect is maximal at 7 days - and the increase we found was as high as 400% in some experiments. Also, importantly, in the experiment of Enquist and his associates the increase in breaking strength correlated directly with the severity of wound infection, they state "Unless overt infection was evident in the experiments, no accelerating effect was seen". This is in marked contrast to our findings, where the accelerating effect was evident in the absence of infection. Also, Smith and Enquist had found that wounds purposely contaminated at operation with Staph. aureus became grossly infected and healing was inhibited. It should be noted that in all their studies Enquist and his associates used silk to close their paramedian incisions. We make our incisions with a single, rapid sweep of the knife, using a subcutaneously placed guide, and close this incision with interrupted fine stainless steel sutures. Edlich and his colleagues have shown that incisions made with a single sweep of the knife, are much more resistant to overt infections than incisions made by the "stop and go" technique, and it is well recognized that silk sutures in contaminated wounds predispose to infection.

Raju, Weiner and Enquist implanted subcutaneously in rabbits stainless steel mesh cylinders treated with sterile culture broth, live E. coli or heat-killed E. coli wound fluid was aspirated by percutaneous puncture daily for 7 days. Similar experiments were carried out in rats with s.c. tunnels into which culture broth, live E. coli or heat-killed E. coli were instilled. They found that the "infected" cylinders and wounds (no histologic or culture studies were reported) were acidotic (lower pH, higher pCO_2) and hypoxic compared to the other two groups. They speculated that "... We might explain the increased strength of the infected wounds as the result of more inflammation, more oxygen utilization, and consequently more fibroblasts and more collagen deposition".

Hunt has pointed out that new blood vessels are stimulated to enter the ischemic, hypoxic, acidotic (including high lactate concentrations) area at the wound edges and that lactate in high concentration stimulates in vitro collagen synthesis, and likely in vivo. No attempt is made in this Progress Report to review this.

Effect of Purposeful Surface Skin Contamination of the Site of Incision Prior to Wounding

We have reported previously our finding that when germfree rats were monocontaminated with Staph. aureus two or more weeks prior to wounding, the rate of gain of wound strength was accelerated. In such an experiment, germfree rats were inoculated with Staph. aureus by oral administration of the staphylococci and then maintained within isolators for two or more weeks as monocontaminants; the Staph. aureus were well established in the gut. The rats were then transferred into a sterile operating isolator and dorsal skin incisions made under pentobarbital anesthesia. The rats were then transferred back into isolators and maintained as monocontaminants postoperatively. Their incisions healed significantly faster than those of their germfree counterparts studied concurrently. (The rate of healing of germfree rats is similar to that of conventional rats; when the incisions of germfree rats are contaminated with Staph. aureus at wounding, healing is accelerated, just as it is in the case of conventional rats). Parallel experiments in which germfree mice were monocontaminated with Staph. epidermidis were carried out; no accelerating effect on wound healing was seen. Cultures of the incisions at sacrifice of the ex-germfree rats monocontaminated orally with Staph.

aureus were positive for Staph. aureus; it is likely that in such ex-germfree mono-contaminated rats the skin is colonized with Staph. aureus and that some of the Staph. aureus are deposited in the incisions during or shortly following operation so that part or all of the observed wound healing accelerating effect may be local rather than systemic.

To explore such a possibility, the clipped dorsal skin of conventional rats was swabbed with an actively growing culture of Staph. aureus 48 hours and 24 hours prior to wounding. Another group was similarly swabbed with an actively growing culture of Staph. epidermidis and a third group with sterile culture medium. The data showed that the pre-wounding swabbing (colonization) with Staph. aureus had a statistically significant accelerating effect (63% increase in breaking strength over that of the control at the 7th postoperative day, $p < 0.001$), no such effect was seen with the Staph. epidemidis. In another experiment, the pre-wounding swabbing of the clipped dorsal skin was carried out 24 hours before wounding. Again, the Staph. aureus pre-wounding swabbing led to an increased breaking strength (41% greater than that of the control, $p < 0.05$, postop. day 7) but the effect was not as great as in the experiment in which the swabbing with Staph. aureus at both 48 hours and 24 hours prior to wounding. This difference may reflect a difference in the degree of skin colonization in the two experiments; this will be tested along with similar experiments in germfree rats to determine whether ther is, as we have hypothesized previously, a greater responsiveness of the germfree rats to the wound healing acceleratino effect of Staph. aureus.

Experiments with local application of non-viable Staph. aureus have shown that the healing of rat skin incisions and the accumulation of reparative collagen in s.c. implanted polyvinyl alcohol sponges are accelerated. These observations will be pursued.

We plan to determine what specific facets of the reparative process are affected by the Staph. aureus and are the basis for its wound healing accelerating effect.

We think it likely that the Staph. aureus exerts itseffect in part by increasing the early inflammatory reaction to wounding, e.g., promoting early infiltration of the wound with cellular, humoral and other key elements (e.g., fibrin scaffolding) to a degree that speed up the succeeding events in wound healing, including endothelial proliferation and new vessel formation, fibroblastic proliferation, and proteoglycan and collagen synthesis. Alexis Carrel 60 years ago and Peyton Rous 35 years ago showed that there may be an optimal degree of intensity in the early inflammatory reaction in terms of healing and that this level of intensity is greater than occurs normally. If the stimulus in increasing the inflammatory reaction is too great, healing may be delayed.

We aim to determine what properties of the Staph. aureus are essential for the wound healing accelerating effect. It is possible that one or more chemicals components of the Staph. aureus and/or a chemical compound(s) produced in the wound as a result of the interaction of the Staph. aureus and the reparative tissue are the active factors.. If so, one may well be able to obtain the wound healing accelerating effect by introducing the appropriate chemical component(s) and avoid the use of viable Staph. aureus. Experiments to pursue this are in progress.

In another aspect of these studies, we are beginning experiments to determine whether the Staph. aureus wound healing effect can ameliorate the impaired healing of seriously injured rats which we have previously demonstrated. In the initial experiments, dorsal akin incisions in rats with unilateral femoral fracture will be the test wounds.

It should be emphasized that the increase in gain of wound strength caused by the purposeful contamination of the incisions with Staph. aureus is considerably greater than has hitherto been reported for any drug, nutrient, or other chemical or physical agent.

VII Additional Studies of Wound Healing

(1) Microcrystalline Collagen Hemostat (MCCH) and Wound Healing

At times following trauma and also during the excision of deep burns bleeding is excessive and difficult to control.

A hemostatic agent, Microcrystalline Collagen Hemostat (MCC) was undergoing preclinical and clinical evaluation by numerous investigators when we started our studies. MCCH (Avitene 1000 (Avicon, Forth Worth, Texas) is purified bovine dermal collagen, shredded into fibrils and converted into an insoluble partial hydrochloric acid salt. The size of the mechanically shredded fibril fragments corresponds to natural aggregates of tropocollagen, that is, they are submicron in size. This fibrillar structure differentiates it from Gelfoam, a gelatin preparation. This new agent has a potent hemostatic action and its general use in clinical surgery was anticipated. Therefore, it seemed important to us to study the behavior, including effects other than hemostatic. We chose in these initial studies to investigate its effects on wound healing in animals and in man and conducted the following studies:

A. Influence of MCCH on the closure of open skin wounds in mice.

B. Influence of MCCH on the healing of skin incisions in rats.

C. Influence of MCCH on the take of split-thickness skin grafts in pigs.

D. Influence of MCCH on the healing of donor sites and the take of split-thickness skin grafts in man.

A. A full-thickness dorsal skin defect was made in mice and standard amounts of MCCH applied immediately to the wound in half the mice. There were no significant differences in wound closure rates between control and MCCH treated mice. B. A standard dorsal skin incision was made in rats. In half the rats, MCCH was put into the incision just before suturing with fine stainless steel sutures. There were no statistically significant differences in breaking strengths between control and MCCH treated rats when tested on the 8th, 20th and 40th days postoperatively. Histologic examination of the wounds showed mild inflammatory reaction surrounding the MCCH-fiber fragments, but no giant cells. Small amounts of MCCH were demonstrable at 40 days. C. Full thickness skin burns in pigs were excised one day after burning. MCCH was applied immediately in some pigs; excess MCCH was removed by saline irrigation. Split thickness skin autografts were applied. MCCH was applied to donor sites. The "takes" of the grafts were excellent and not affected by the use of MCCH and the donor sites healed uneventfully. D. MCCH was used in four patients with burns, three of whom underwent early excision of full thickness skin burns and immediate autografting. The fourth underwent skin grafting to the granulating areas 3 months after injury. The MCCH was applied to some donor sites and to some areas of excision. In one patient with severe burns, wound sepsis developed equally in areas with and without MCCH and the grafts were lost. In the other three patients, there were excellent takes of the grafts at all sites. All donor sites, treated and untreated, healed normally in all patients.

(2) Effect of Cimetidine on Wound Healing

Cimetidine is the most prescribed drug in the world and it is used commonly in seriously injured patients to help prevent stress ulceration. There has been concern that it may interfere with wound healing. This concern stems from evidence which suggests that endogenous nascent histamine stimulates wound healing. Recently, it has been shown that exogenous histamine and 4-Methistamine (an H-2 agaonist) injected into wounds have a stimulatory effect in low doses and an inhibitory effect in high doses on reparative collagen accumulation. These effects were inhibited by prior local administration of cimetidine (an H-2 antagonist), but not by mepyramine (an H-1 antagonist), suggesting that histamine's effect on reparative collagen accumulation may be mediated through activation of H-2 receptors.

Because cimetidine is used very frequently for the prevention of stress ulceration in seriously injured patients, we conducted an experiment to determine whether cimetidine given orally inhibits wound healing. Sprague-Dawley rats (300-400 g) were randomly divided into 2 groups of 26 each: Group A rats were fed a standard laboratory rat chow; Group B rats fed the same chow supplemented with cimetidine, 300mg/kg diet. All rats ate and drank water ad libitum. Ten days later under pentobarbital anethesia our standard 7 cm paravertebral skin incision was made in each rat and a polyvinyl alcohol sponge implanted s.c.; the incisions were sutured with interrupted fine stainless steel sutures. The animals were maintained on their respective diets and 15 of each group killed on the 5th post-operative day and 11 of each group killed on the 10th postoperative day. There were no statistically significant differences in the breaking strengths or hydroxproline contents of the sponges in the two groups of rats at either time

We conclude that cimetidine in doses comparable to those used clinically does not inhibit wound healing in rats.

(3) Effect of Thymosin on Wound Healing

The observation of Dougherty of Utah that the administration of thymosin to mice increases the number of fibroblasts in the loose areolar tissue suggested to us that thymosin might have an accelerating effect on wound healing.

A series of in vitro and in vivo experiments to establish the effects of thymosin on fibroblastic proliferation and wound healing was carried out in collaboration with White and Goldstein using a preparation of thymosin as prepared by an earlier method of theirs.

Parallel groups of mice with and without thymosin injections were studied; the thymosin was dissolved in 0.9% NaCl; the controls were injected with the saline alone. Mice were used rather than rats because mice are more responsive to thymosin. Standard skin incisions and polyvinyl alcohol sponge implants (subcutaneous) were made; body weights, food and water intakes were followed. At varying time postoperatively (2-20 days) the animals were killed.

There was a significant systemic thymosin effect in terms of increase in lymph node size (axillary,inguinal), rate of incorporation of ^{3}H-thymidine into lymph node DNA, and number of peripheral lymphocytes. There was also a significant increase in the thymosin treated animals in the number of inflammatory cells in the reparative tissue in both the skin incisions and sponge granulomas in the first week postoperatively as judged nistologically, by DNA contents, and uptake of ^{3}H-thymidine into DNA, as compared with control mice receiving saline, the vehicle in which the thymosin was dissolved.

Later, after the inflammatory phase passed, there was little difference between the thymosin treated and untreated mice in the healing of skin incisions assessed by the histologic appearances and the breaking strength of the wounds, and the histologic appearances and hydroxyproline contents of the sponge reparative tissues 7-20 days postoperatively. Thymosin, thus, induced an increased in the inflammatory reaction to wounding.

(4) Effect of Burn Wound Sepsis on Healing of Skin Incisions

An experiment was carried out to determine how a developing infection of a 3° back burn might affect the healing of a ventral skin incision. For this, a group of male Sprague-Dawley rats weighing about 360g were divided randomly into four groups of 8 rats each. Group 1 was not burned and nothing was applied to the skin of the back, group II was burned and the burn was swabbed with sterile culture medium, groups III and IV were burned and the burns were purposely contaminated by topical application of an actively growing culture of Ps. aeruginosa which we had shown in other experiments to be pathogenic for rats; for group III, $5x10^3$ Ps. aeruginosa were applied; for group IV, $5x10^5$ Ps. aeruginosa were applied. The 3° burns were produced by dipping the backs (10% body surface) of the rats (anesthetized with ether) into 100°C water for 9 seconds; group I rats were anesthetized and their backs dipped into water at room temperature for 9 seconds. A seven cm ventral skin incision was then made, sutured with interrupted fine stainless steel sutures, the animals placed prone and the burns of the four groups of rats treated as described. All rats ate our standard rat chow and drank tapwater, both ad libitum.

The data showed that while group I rats (unburned, wounded) gained substantial weight in the next 12 days (from 361±4 g preoperatively to 396±3 g), all groups of burned rats just maintained their body weights; in each case, the difference compared to group I was highly statistically significant. However, there were no differences among any of the groups in the breaking strengths of the incisions, tested both in the fresh state and after fixation in 10% buffered formalin. In this experiment, then, a developing non-lethal burn wound sepsis (a 3° burn of the back which involved 10% body surface) prevented body weight gain but did not impede the gain of strength of a distant (ventral) skin incision. We know from previous experiments that much larger 3° burns (33% body surface), uninfected, impair wound healing of laparotomy wounds of rats (Levenson, S.M., Pirani, C.L., Braasch, J.W., Waterman, D.F., Surg. Gynecol. & Obstet. 99: 74, 1954) and guinea pigs (Levenson, S.M., Upjohn, H.L., Preston, J.A., Steer, A., Ann. Surg. 146: 357, 1957).

VIII Thymosin: Bacterial and Fungal Infection

In our initial experiments aimed at determining the possible effects of thymosin administration on altering the response of animals to wound infection, the burned tails of mice were dipped into a culture of Ps. aeruginosa highly virulent for mice, a technique described by Sanford Rosenthal. Some mice received thymosin daily, beginning at the time of challenge, some received the saline vehicle in which the thymosin was solubilized, some were given neither. The mortality in several replicate experiments was prompt, very high, and similar in both groups. In another early experiment, we looked at the effect of thymosin administered to mice whose systemic resistance to infection was decreased by a single injection of cortisol and who were then challenged with Candida albicans, injected intravenously, in a dose which was lethal for 80-90% of control mice receiving saline. Thymosin in low dosage had no influence or this mortality.

These experiments were carried out several years ago. Since then there has been significant advances in the preparation of thymosin and increased information about dosage requirements. Also, there is now a much greater supply, so experiments can be conducted in rats and guinea pigs as well as mice.

IX. Some Additional Studies of Stress Ulcer and Other Stress Responses

Choline: Anti-Stress Properties

a. Inhibition of Restraint Ulceration by Supplemental Choline

Ischemia of the gastrointestinal mucosa is thought to be involved in the pathogenesis of stress gastrointestinal ulceration and the ischemia may be due to sympathetic nervous activity. The vascular response of tissues to the catecholamines is modulated by the ratio of catecholamines: cholinergic compounds. As a result, some cholinergic mediators or agonists diminish some key target organ responses to adrenergic (sympathetic) stimulation. We had shown several years ago, that the nephropathy which occurs in weanling male rats fed a choline deficient diet was likely due to an interference with renal blood flow due to an imbalance between catecholamines and acetylcholine: (a) there was an abrupt drop in renal and brain acetylcholine in choline deficiency; (b) the mesenteric circulation of choline rats deficient rats was markedly reactive to topical epinephrine.

We hypothesized that choline would inhibit stress gastrointestinal ulceration. To test this, four groups of male Sprague Dawley rats (260g) were used. Group A (controls) was maintained on basal rat chow for 4 2/3 days. Group B (fasted) was maintained like Group A for 3 days and then starved for 1 2/3 days. Group C was maintained like Groups A and B for 3 days, starved for 16 hours (2/3 day), then subjected to total body restraint and continued fasting for 1 day. Group D animals were treated like Group C except their drinking water consisted of a 0.3% solution of choline chloride. The rats were killed 4 2/3 days after the start of the experiment. The results are shown in the following table.

	No.of rats with gastric ulcers	No.of ulcers	No.of large gastric ulcers	No.medium sized gastric ulcers
Group C (fasted & restraint)	9/10	23	8	6
Group D (fasted & restraint, choline supplemented)	5/10	9	3	0
Group A (no fasting, no restraint)	0/10	0	0	0
Group B (fasted, no restraint)	0/10	0	0	0

In a second experiment, similar results were obtained. This finding that choline inhibits this form of stress ulceration supports but does not prove our hypothesis.

b. Effect of Supplemental Choline on the Adrenal Enlargement and Thymic Atrophy of Stress

Recent work has emphasized the importance of catecholamines as important mediators of the metabolic response to trauma. Wilmore, et al. (Surg.Gyn.Obs. 138:875,1974) have shown partial blockade of the hypermetabolic response by β adrenergic blockade

agents. We have found that choline supplementation (0.3% added to the drinking water or to a commercial laboratory chow adequate for growth, development and reproduction of normal mice) has a marked thymotropic effect ($p < 0.001$) in unstressed mice and prevented the adrenal enlargement and thymic involution in stressed mice.

In a typical experiment, young C3H/Hej female mice were divided into two groups: Group A drank tap water and ate our standard lab chow which is not choline deficient and which, as noted, permits normal growth, reproduction and longevity of normal mice. Group B ate the same diet but drank a 0.3% solution of choline chloride in tap water. Choline supplementation was begun 3 days before the casting. All mice ate and drank ad libitum. After 3 days, half the mice in each group were subjected to partial body casting. Three days later all were killed. A similar experiment was conducted using female Balb/c mice. The data are shown in the following table 13.

Table 13

	Exp. 1 C3H/Hej female mice				Exp. 2 Balb/c female mice			
Wt. (mg)	Unstressed		Stressed		Unstressed		Stressed	
	Chow	Choline	Chow	Choline	Chow	Choline	Chow	Choline
Thymus	28.1±1.0	30.1±1.1	20.3±1.0	25.0±1.2	38.5±2.5	48.0±3.3*	20.2±1.4	29.3±2.4**
Adrenals	3.3±0.2	3.2±0.2	5.2±0.2	3.6±0.2*	4.1±0.3	3.5±0.2	5.1±0.3	4.2±0.2*

*, $p < 0.001$; **, $p < 0.02$

Choline increased thymic weight in unstressed Balb mice as indicated in the table. In stressed C3H and Balb mice choline prevented adrenal hypertrophy and thymic involution. Since many aspects of stress are mediated via the adrenergic nervous system and since these may be modulated by parasympathetic activity, we think it likely that choline's action is due to its cholinergic properties and to those of its metabolite, acetyl choline.

Supplemental choline also increased the resistance of mice to the Moloney sarcoma virus ($p < 0.05 - < 0.001$). The supplemental choline also prevented the adrenal enlargement, thymic involution, and decreased resistance to tumor growth that charactertistically follows partial body casting (Table) and/or inoculation with the Moloney sarcoma virus ($p < 0.001$ in each case). The choline supplementation was begun three days before the viral inoculation in the various experiments. This experiment was carried out as a test of the possible effectiveness of choline in prevention of a viral infection, not as a study of tumor prophylaxis per se.

We suggest that supplemental choline modifies the release or action of catecholamines on metabolic responses to stress in much the same way as choline inhibits the action of catecholamines on heart rate and vasoconstriction.

X. Liver Regeneration

A. Introduction

The ability of the lvier to regenerate rapidly after partial hepatectomy is striking. Thus, after two-thirds of the liver is resected in the rat, the liver remnant reaches the preoperative size of the organ within 2 weeks and then stops. A similar, but substantially slower, process occurs in man following partial hepatectomy because of severe injury to the liver (most often of the right lobe), an important form of war (and civilian) injury, or because of limited hepatic neoplasms. Conceptually, the process from some points of view, is similar to that of a healing wound: (1) What initiates the healing and regenerative processes? (2) What keeps them going? (3) What stops them?

We have been interested in the problem of liver regeneration in relation to wound healing and injury for some time. While at the Walter Reed Army Research Institute, we found that while the healing of skin incisions was impaired in rats with severe burns, liver regeneration following partial hepatectomy was accelerated in such rats. These physiologic data correlated with studies of protein synthesis and breakdown which we conducted using N^{15} glycine in these animals (1).

B. Background

The thought that the sera of animals following partial hepatectomy contain factors influencing liver regeneration in primary and secondary ways has been held for a long time. Experiments using tissue culture techniques to compare the effects of sera obtained from animals before and after partial hepatectomy have shown that fibroblastic outgrowth from liver explants and the duration of survival of cultured fibroblasts were greater when the culture medium contained serum from partially hepatectomized rats rather than serum from normal rats (Glinos and Gey, 1952). These experiments followed those of Bucher and associates (1951) which showed that when one member of a pair of parabiotic rats underwent partial hepatectomy, the unoperated partner developed an increased hepatocyte mitotic rate. There have been a great many studies following up these observations: we will not review them here since we have reviewed them in previous Progress Reports and they have been reviewed extensively by others in a number of Symposia and books.

The possibility exists that a factor(s) appears in the serum after partial hepatectomy which stimulates growth of the hepatic cells. Alternatively, a factor(s) preventing the growth of liver cells in the normal animal with an intact liver may disappear from the serum following actual hepatectomy, or, indeed, both the occurrence of stimulatory and the disappearance of inhibitory factors may occur. Both in vivo and in vitro studies have been made in an attempt to demonstrate humoral agents related to liver regeneration.

It is generally believed that the stimulus for hepatic regeneration is tissue specific. There is however experimental evidence suggesting that this may not be so. Cardoso and his associates studied the effect of partial hepatectomy upon the circadian distribution of mitosis in the cornea of rats and found evidence of increased mitotic activity following partial hepatectomy. Paschkis and his associates reported similar findings. A stimulatory effect on tumor growth following partial hepatectomy was also observed by Trotter who studied subcutaneously transplanted hepatomas in mice. Gentile, Ali and Grace found that serum from rats 24 hours following partial hepatectomy, in concnetrations of 0.6 to 5% stimulated the growth of primary cultures of embryonic rat fibroblasts. Sakai, Pfefermann and Rountz have recently reported experiments indicating that serum obtained from rats subjected to 70% partial hepatectomy stimulated both hepatocytes and lymphocytes in culture. Bucher and Malt also believe that the non-specific effects that have been reported are slight and do not challenge the notion that the hepatotropic factor acts primarily on the liver cells.

As described in the preceding section there are reports to suggest that a) a factor appears in the serum of hepatectomized animals which stimulate liver regeneration, b) a factor is present in the serum of normal rats which inhibits regeneration, and c) both a) and b) are true. Further, evidence has been obtained indicating that certain hormones (e.g., insulin, glucagon) modify the rate of liver regeneration but that liver regeneration can begin in their absence albeit at a substantially slowed rate. Our aim was to clarify some aspects of these matters.

One of the central techniques employed in our studies was to determine the effects of sera and sera ultrafiltrates from rats and humans on the growth in tissue culture of E_1 cells, hepatocytes derived from the foetal rat liver, which we obtained from Dr. Harry Eagle at the Albert Einstein College of Medicine. The results of our experiments have been published in a number of papers. We studied sera of rats, dogs, and humans under a wide variety of conditions.

We interpret our studies as indicating the following:

C. Our Studies in Rats and Patients

1) a serum factor(s) inhibiting liver regeneration is not present in normal rats or humans;

2) a serum factor(s) stimulating liver regeneration is present following partial hepatectomy in rats and humans;

3) neither total portal vein gradual occlusion in rats, nor 99.5% pancreatectomy in rats, nor unilateral nephrectomy in rats or humans affect the appearance and activity of this serum factor(s) following partial hepatectomy;

4) this stimulating factor(s) is ultrafilterable and has a MW of 5,000 - 12,000;

5) glucagon and insulin each stimulate the growth in tissue culture of E_1 cells when added to media containing sera from normal or sham-hepatectomized rats. These effects are much less when the insulin and glucagon are added to media containing sera from rats with partial hepatectomies. It is of interest that insulin and glucagon have similar effects in this system, whereas in many physiological situations they act antagonistically;

6) although insulin and glucagon modify the rate of liver regeneration, it is unlikely in our view that they are primary initiators of this process. The hepatotropic activity of sera from rats following hepatectomy as assessed by the growth of E_1 cells in culture is independent of its insulin content, since serum from rats undergoing partial hepatectomy after 99.5% pancreatectomy and maintained on small doses of oral insulin has undiminished hepatotropic activity despite very low levels of serum insulin, levels which are the same low values seen in rats with 99.5% pancreatectomy and sham-hepatectomy whose serum has no hepatotropic activity.

The effects of sera of rats 24 hours following 68% partial hepatectomy or sham-hepatectomy on hepatocyte growth in vitro were tested a) directly, b) after treatment with insulin-specific antibody, and c) after treatment with charcoal which binds both immuno-reactive insulin and glucagon. Bound hormones in (b) and (c) were removed by centrifugation. Hepatocyte stimulation was assessed by ^{3}H-thymidine incorporation in heaptocyte DNA, total hepatocyte DNA, and hepatocyte ornithine decarboxylase (ODC) activity. For these measurements, we used primary monolayer cultures of hepatocytes. Rat hepatocytes were obtained from adult male rats. The livers were perfused in situ according to the method of Bonney (In vitro, 9: 399 (1974)), and monolayer cultures were established. The cells were grown in Waymouth's medium MB M52/1 and 5% fetal calf serum at 37° in a 95% air and 5% CO_2 atmosphere. The serum to be tested was added to the medium (5% concentration) and incubated for 48 hours. The ^{3}H-thymidine was then added and 6 hours later measurements made of the number of viable hepatocytes, incorporation of ^{3}H-thymidine (New England Nuclear, Boston, MA) into TCA-insoluble material of the hepatocyes, hepatocyte DNA, and hepatocyte ornithine decarboxylase activity. Serum insulin and glucagon were 15.7±2.0 μU/ml and 243±29 pg/ml respectively in sham-hepatectomized rats and 10.2±1.3 μU/ml ($p < 0.001$) and 930±45.2 pg/ml ($p < 0.001$) respectively in partially hepatectomized rats. The rate of thymidine incorporation into hepatocyte DNA was 12 times faster ($p < 0.0001$) and the ODC activity was 34 times greater ($p < 0.0001$) when the hepatocytes were cultured in media containing sera from rats subjected to partial hepatectomy. This striking hepatotropic activity persisted at the same high level after insulin was selectively completely removed by insulin antibody without affecting the glucagon concentration and after both insulin and glucagon were completely removed by the charcoal treatment.

Recently, we have conducted a similar experiment, testing in addition the possibility that the fetal calf serum which is one of the ingredients in the basal culture medium might be contributing some insulin and/or glucagon or some other hepatotrophe. For this, in the repeat set of experiments, assays were conducted using the following:

(1) Serum from rats 24 hours after 68% partial hepatectomy,
(2) Serum from rats 24 hours after sham-hepatectomy,
(3) Serum from rats 24 hours after 68% partial hepatectomy treated with charcoal,
(4) Serum from rats 24 hours after sham-hepatectomy treated with charcoal.
(5) Fetal calf serum,
(6) Fetal calf serum treated with charcoal.
(7) Fetal calf serum treated with insulin-specific antibody. At the time of this experiment we had very limited insulin-specific antibody: we chose to use it for the fetal calf serum because we already had information on the effects of treatment with the insulin-specific antibody of serum from rats with sham-hepatectomy and from rats with partial hepatectomy - in each of those cases, selective removal of the insulin did not affect the widely differing effects of these latter two sera on the hepatocytes in vitro.

The results of this recent experiment showed that the effects of the fetal calf serum before and after treatment with the insulin-specific antibody or with the charcoal on (^3H) thymidine incorporation into hepatocyte DNA and on hepatocyte ornithine decarboxylase activity are similar to that of the sera from the rats undergoing sham-hepatectomy and that the effects of both of these are significantly different from the highly stimulatory effects of the sera from rats which underwent partial hepatectomy.

The results of this experiment confirmed also our previous finding that removing all the insulin and glucagon from the sera of the rats which had undergone partial hepatectomy had no effect on the *in vitro* hepatocyte stimulatory effects of such sera.

8) the cellular and tissue specificity of the hepatotropic factor(s) is still unsettled; we found that sera from partially hepatectomized rats accelerated not only the growth of E_1 cells (hepatocytes derived from foetal rat liver) but also the growth of fibroblasts derived from rat embryomic tissue but not fibroblasts derived from the skin of adult rats;

9) the concentrations of certain amino acids may modify the rate of liver regeneration, but they are not the primary hepatotropic factor(s). Following partial hepatectomy (70%) in rats there is a prompt change in most of the serum amino acid concentrations. Most amino acids increase, generally by a factor of 1.5 - 2. Ornithine goes up 4-fold while arginine falls slightly and alanine is unchanged. We tested the effect of varying amino acid mixtures on the growth of E_1 cells in culture by adding supplements of these mixtures to the basal culture media. We found that the mixture simulating the changes in serum amino acid concentrations 4 hours after hepatectomy accelerated the growth of the E_1 cells while mixtures simulating the serum amino acids of normal rats or rats undergoing sham-hepatectomy had no such effect. Such changes in amino acid concentrations are not the basis for the action of the serum hepatotropic factor(s) we have demonstrated since the hepatotropic factor(s) has a molecular weight 5,000 - 12,000 and thus substantially larger than the free amino acids. Leffert has found that arginine and ornithine are obligatory amino acids for the growth of hepatocytes in culture.

10) Ornithine Decarboxylase as an Early Indicator of *in vitro* Hepatocyte DNA Synthesis

The enzyme ornithine decarboxylase, one of the key enzymes involved in [illegible] amine biosynthesis, catalyzes the decarboxylation of ornithine to yield putrescine. The activity of this enzyme in an *in vitro* hepatocyte culture assay system was measured because it is known that ornithine decarboxylase levels increase in instances where active protein synthesis, DNA synthesis, and cell growth is initiated. We found a good correlation between ornithine decarboxylase activity and the rate of tritiated thymidine incorporation into hepatocyte DNA. The increase in enzyme activity precedes the incorporation of tritiated thymidine into DNA (enzyme activity increases 2-3 hrs. following stimulation of cell growth; whereas the tritiated thymidine uptake increases at about 14-18 hours). These experiments were done using rat hepatocytes which we obtained from adult male Sprague-Dawley rats which had undergone no operation, sham-hepatectomy or 70% partial hepatectomy under ether anesthesia. Sham-hepatectomies included a midline abdominal incision and palpation of the liver under ether anesthesia. Blood samples were obtained by cardiac puncture under ether anesthesia 24 hr. postoperatively. The livers were perfused *in situ* according to the method described by Bonney (*In vitro* 10: 130, 1974) and monolayer cultures were established. The cells were grown in Waymouth's medium and 5% fetal calf serum at 37°C in 5% CO_2 and 95% air atmosphere. The sera to be tested were added to the cells in 5% concentration.

We found that addition of serum from sham-hepatectomized rats to the tissue culture medium did not result in a statistically significant change in or the rate of incorporation of tritiated thymidine into DNA. However, a significant (13-fold) increase in tritiated thymidine incorporation into DNA was obtained when serum from animals which underwent partial hepatectomy 24 hr. previously was added to the culture medium. Similarly, only a relatively modest increase in the activity of ornithine decarboxylase was seen when serum from sham-hepatectomized animals was added to the culture medium whereas addition of serum from partially hepatectomized rats to the culture medium resulted in a very large (18-fold) increase in enzyme activity. The increase in the activity of ornithine decarboxylase occurred as early as 2 hours following addition of serum from partially hepatectomized animals to the culture medium. The increase in enzyme activity was maintained up to 6 hr. and returned to the normal level in about 8 hr.

D. Some Ancillary Studies Concerning the Possible Source(s) and Nature of the Serum Hepatotropic Factor(s)

Does the regenerating liver produce an hepatotropic factor(s)?

In other experiments we used the approach of testing the effect of "conditioned" tissue culture media on the growth of hepatocytes in vitro. The term "conditioned" refers to media in which cells have been growing in culture and then the cells removed and the media used to assess the growth of newly added cells.

Koch and Leffert had found that serum-deficient conditioned serum obtained from primary cultures of fetal rat hepatocytes initiates DNA synthesis and mitossi in homologous quiescent monolayer cultures. The effect may be tissue-specific since they found that serum-deficient conditioned media from 3T3 fibroblast cultures was inactive for the fetal rat hepatocytes. Arginine and a lipid or lipid containing material were shown to be two of the active factors in the conditioned medium obtained from the fetal rat hepatocytes.

For our experiment, rat hepatocytes were obtained from adult rats which had undergone no operation, sham-hepatectomy, or 70% hepatectomy 24 hours previously. The livers were perfused in situ according to the method of Bonney (In vitro 10: 130 , 1974) and monolayer cultures were established. At several time intervals the media from the normal liver cells were substituted with "conditioned media" from 70% hepatectomized, sham-hepatectomized, and other normal liver cells.

We found that twelve-hour conditioned media from regenerating liver cells, when added to normal hepatocyte monolayer cultures, produced a great simulation (ninefold) of tritiated thymidine into the DNA of normal hepatocytes 14 hour later . Media conditioned with hepatocytes obtained from sham-hepatectomized rats had a modest effect on the rate of incorporation of tritiated thymidine to the DNA of normal hepatocytes. Twelve-hour conditioned media from regenerating liver cells, when added to normal hepatocyte cultures, produced a very large (13-fold) stimulation of ornithine decarboxylase activity 4 hr. later. A modest increase in enzyme activity was obtained with media conditioned for 12 hr. with normal hepatocytes or with hepatocytes from sham-hepatectomized rats. Because of the possibility that the stimulatory effect of conditioned media with regenerating liver cells may be due to increased nutrient concentration in the media due to possible underutilization by the regenerating cells, controls were included in which the effect of addition of fresh media on ornithine decarboxylase activity and tritiated thymidine incorporation into DNA was studied. The addition of fresh medium to normal hepatocyte monolayer cultures produced a significant but relatively modest (two-fold) increase in ornithine decarboxylase activity and a minimal increase in tritiated thymidine incorporation into DNA which was not found to be statistically significant. Both of these effects were much less pronounced than those seen when media conditioned with regenerating liver cells were added.

In summary, we measured the activity of ornithine decarboxylase in our tissue culture system with the view that it would serve as a method to assay early hepatocyte DNA synthesis and initiation of cell growth. Our results show that ornithine decarboxylase activity can indeed be used for this purpose. Use of this enzyme assay has several advantages over measurements of tritiated thymidine incorporation into DNA: (a) the enzyme assay is technically simpler and faster than the tritiated thymidine uptake assay; (b) the increase in enzyme activity precedes the increase in the incorporation of tritiated thymidine into DNA (enzyme activity increased 2-3 hr following stimulation of cell growth, whereas the tritiated thymidine uptake increases at about 14-18 hr). Using conditioned media, we showed that hepatocytes derived from the regenerating liver remnant secrete a factor(s) into the culture medium which then added to normal hepatocyte monolayer cultures produces marked stimulation in the activity of ornithine decarboxylase and tritiated thymidine incorporation into DNA. No such dramatic stimulatory effect was seen when media conditioned with normal rat hepatocytes or hepatocytes from sham-hepatectomized rats were added to normal hepatocyte monolayer cultures.

In follow-up experiments, we isolated and considerably purified a factor from regenerating liver which stimulates hepatocytes in vitro. The assay system used consisted of normal rat hepatocyte monolayer cultures in which total cell DNA, ^{3}H thymidine incorporation into DNA, total protein, number of cells and ornithine decarboxylase activity measured. The isolation and purification was accomplished by chromatography on a Sephadex G-75 column followed by DEAE-52 cellulose chromatography and then further chromatography on a hydroxyapatite column. One major protein peak with a significant ($p < 0.001$) stimulatory activity was obtained. The final fraction (8% yield) was 8,000-fold purified. The stimulatory effect appears to be specific for hepatocytes. When livers from normal unoperated rats and sham-hepatectomized rats were carried through the same purification procedure, similar peak patterns were obtained, but the specific activity of all fractions was very low. The final purified fraction from the livers following partial hepatectomy had a 4,000-fold greater specific activity than the final purified fractions from normal and sham-hepatectomized livers.

Disc polyacrylamide gel electrophoresis of the final fraction revealed a single band. Sephadex G-200 chromatography showed a single protein peak which coincided with biological activity. A molecular weight of about 42,000 was determined by Sephadex G-200 chromatography. The hepatocyte stimulatory material from regenerating liver is heat-stable, retains activity following pre-treatment with DNAse and neuraminidase, but loses its activity following pre-treatment with trypsin and chymotrypsin. We had found in other experiments that the activity of the serum hepatotropic factor(s) which appears following partial hepatectomy was also lost (or greatly diminished) after treatment of the serum ultrafiltrate fraction (5,000-12,000 MW) with trypsin.

In additional experiments we found that when the highly purified fraction obtained from rat regenerating liver (following 70% hepatectomy) was added to normal rat hepatocytes in monolayer cultures, there was a 10-fold increase in the total DNA per tissue culture plate ($p < 0.001$), an increase in the rate of incorporation of tritiated thymidine into DNA ($p < 0.001$) and a 100-fold increase in the activity of the enzyme ornithinedecarboxylase, a sensitive indicator of the degree of stimulation of cell growth ($p < 0.001$).

The amount of hepatocyte stimulating factor protein required to produce a 10-fold increase in the total amount of DNA per tissue culture plate in vitro was defined as one unit of biological activity. We found that the intraperitoneal injection of 5 units of this highly purified fraction into 30 male adult normal Sprague-Dawley rats resulted in a highly significant ($p < 0.001$) increase in the activity of ornithine decarboxylase in the normal rats liver at 10 hours following injection. Significant ($p < 0.001$) increase in total liver DNA at 20 hours after injection and a 18-fold increase in the rate of uptake of tritiated thymidine into liver DNA ($p < 0.001$). Thus, this factor which we have isolated and highly purified from regenerating rat liver is highly active both in vitro and in vivo.

These data suggest that hepatocytes from regenerating liver produce a factor(s) which stimulates normal hepatocyte growth in vitro, a view also held by other investigators as a result of their investigations, including Levi, Zeppa, Ann. Surg. 174 364 (1971)); Ruhenstroth-Bauer, Golberg, Silz, et al., Hoppe-Seyler's Z. Physiol. Chem. 359:543 (1978)); LaBrecque, Pesch, J. Physiol. 248: 273 (1975)); Starzl, Terblanche, Porter, Lancet 1: 127 (1979)); van Hoorn-Hickman, Kahn, Green, et al., Hepatology 1 287 (1981)). Fausto, Brandt and Kestner have expressed the view "The biochemical adaptations occurring in the liver fragment as a response to a decrease in organ mass is seen as the primary stimulus for the growth of the liver after partial hepatectomy."

E. Our Studies in Dogs

1) Sera from dogs, normal, sham-hepatectomized, and partially-hepatectomized (30 and 60%) behave in ways comparable to those of parallel groups of rats, that is, we found no evidence of stimulator(s) or inhibitor(s) in the sera of normal or sham-hepatectomized dogs, but we did find evidence of stimulator(s) in the sera of dogs obtained 7-14 days, following partial hepatectomy, as assessed by the growth of E_1 cells in culture.

2) We had previously reported on experiments in which rats were subjected to 99.5% pancreatectomy and found that the sera of such rats did not stimulate the growth of E_1 cells in culture, but the sera of such rats which had a subsequent partial hepatectomy (70%) did stimulate the growth of E_1 cells, and to the same extent as rats with intact pancreases subjected to partial hepatectomy. However, 99.5% surgical pancreatectomy in rats is a very difficult technical procedure because of the nature of the blood supply to the duodenum and some of the rats operated upon may develop ischemic necrosis of the duodenum, a factor which complicates such a study. By contrast, total pancreatectomy in the dog is a technically simple procedure. Further cannulation of various vessels for acute, sub-acute, and chronic experiments is much easier in the dog than in the rat.

In our initial studies, we found that serum of a dog subjected previously to partial hepatectomy (30%) three months following total pancreatectomy stimulated the growth of E_1 cells in culture to the extent as sera from dogs with intact pancreases subjected to similar (30%) hepatectomies. The pancreatectomized dogs had been kept on insulin lente until three days prior to partial hepatectomy when the insulin was stopped. We have shown previously in this same dog that within 2 days following the last injection of the insulin lente, immunoreactive insulin was not detectable in the peripheral blood; in this experiment, the dog was without insulin for 7 days.

(continued next page)

F. Influence of Clofibrate and Nafenopin on Liver Growth and Regeneration

Although both of these compounds have been shown by others to cause liver enlargement in rats, the type of enlargement induced has not been fully described. Investigators at our institution found increased mitotic activity in the livers of rats fed Nafenopin. This led us to explore the possible utility of Nafenopin and related compounds (Clofibrate) as stimuli for liver regeneration.

In an initial experiment, groups of male Sprague-Dawley rats weighing 270 g were fed either a) a standard laboratory chow, b) the same chow containing 2 g Clofibrate/kg diet or c) the same chow to which 1 g Nafenopin/kg diet was added. Three days later some animals from each of the three groups were subjected to 30% hepatectomy, the others in each group were continued as unoperated controls. Five days later (8 days after the commencement of the feeding of the experimental diets) the animals were killed and the livers were removed for analyses.

Liver weights were increased by the ingestion of Clofibrate (17%) and Nafenopin (55%), especially the latter, in both the unoperated rats and those subjected to 30% partial hepatectomy. The increases in liver size in each case represented increases in liver protein, the proportions of liver water and fat were the same in all groups. The relative increase in gain of liver mass after partial hepatectomy, was influenced by Clofibrate (41%) and Nafenopin (7%) to an apparently greater extent than occurred in the livers of unoperated rats. The proportions of liver water, fat and protein were the same in all groups.

There were two aims involved in a second experiment: (1) to confirm the Clofibrate effect on liver regeneration, (2) since partial hepatectomy is frequently an emergency procedure, we wanted to determine the effect of administering Clofibrate beginning the day of partial hepatectomy. The rats were divided into 3 main groups, unoperated, sham-operated, and 30% partial hepatectomy. Each of these groups was subdivided into two groups, one receiving Clofibrate in the diet, the other, not. All rats were killed six days after the [illegible] procedures.

The results showed that Clofibrate again caused an increase in liver size in all groups. The increase in all cases is due to liver growth. The ratios of DNA/liver wt. and RNA/liver wt. did not alter relative to the paired control groups. The increase in liver size was not due to excess fat or water accumulation in any group treated with Clofibrate since the proportions of fat, water, and protein were not altered. These results show that Clofibrate begun right after hepatectomy increased liver growth but the proportionate increase was less than when the Clofibrate was given prior to hepatectomy as in the first experiment.

In a third experiment, five groups of CBA male mice were studied:

A-unoperated, chow diet, B-unoperated Nafenopin (2g/kg diet), C-sham operated, Nafenopin (2 g/kg diet), D-30% hepatectomy, chow diet, E-30% hepatectomy, Nafenopin (2 g/kg diet)

Feeding of Nafenopin was begun right after hepatectomy as in the second experiment with rats. Half of each group of mice was killed after 24 hours, the other half at 48 hours.

We found that Nafenopin increased liver size in all 3 groups of mice; this effect was evident in 24 hours and increased in the second 24 hours.

G. Clofibrate and Wound Healing

There is some evidence to indicate that under certain conditions fibroplasia is limited by the size of the proline pool, which in turn is dependent on hepatic synthesis of the amino acid. Since Clofibrate increases liver mass (DNA, RNA, protein) we wondered whether the Clofibrate might in turn influence wound healing through its effect on the liver. Two groups of Sprague-Dawley male rats weighing 380 grams were used. They were subjected to 7 cm. dorsal skin incision and s.c. implantation of a single polyvinyl alcohol sponge. On the day of wounding, one group was begun on a chow diet containing 3 g Clofibrate/kg diet; the other group was continued on the chow diet. On the 10th postoperative day, the rats were killed, the breaking strengths of the incisions measured and the reparative granulation tissue in the sponges analyzed for hydroxyproline.

We found that Clofibrate again increased liver size, 13.0 g vs 19.6 g, $p < 0.0001$. This increase is greater than we observed in other experiments; this is probably related to the large dose of Clofibrate used in this experiment (3 g/kg diet vs 2 g/kg diet). Kidney size was also increased by Clofibrate (3.4 g vs 3.0 g, $p < 0.001$). Clofibrate did not affect wound strength significantly but did cause an increase in the hydroxyproline content of the sponge reparative granulation tissue (594 ug/100 mg dry sponge vs 498 µg/100 mg dry sponge, $p < 0.001$).

xI The Effect of Ethanol and Its Metabolites on Collagen Content and Fibroplasia in vitro

Alcoholic liver damage is an all too frequent medical problem. Previous studies have shown that acetaldehyde, an intermediate metabolite of ethanol, increases collagen synthesis when added to fibroblast cultures. It has been proposed that acetaldehyde is the direct stimulus for collagen synthesis in alcohol-induced cirrhosis. We hypothesized that other metabolites of ethanol may affect fibroplasia and collagen accumulation. To test our hypothesis we harvested rat liver fibroblasts following portal vein collagenase perfusion. Cells were maintained at 37°C, 5%, CO2, in DMEM plus 10% FCS and antibiotics. Cells were divided into the following groups: control, 5.0 mM ethanol, 50 mM acetaldehyde, 50 mM sodium acetate, and 50 um acetyl CoA. Cells were harvested daily for cell counts and determination of viability. Flasks from each group were assayed for collagen content by measuring the hydroxyproline content colorimetrically (Woessner). Ethanol and its metabolites had no effect on cell growth. There was an increase in collagen content following addition of acetaldehyde ($p < 0.02$) and no significant effect when ethanol or sodium acetate were added to the cells; however, there was a striking increase in collagen content when acetyl CoA was added to the fibroblasts ($p < 0.01$).

In conclusion, we confirmed that acetaldehyde stimulates accumulation of collagen in liver fibroblast cultures and showed that another ethanol metabolite acetyl CoA, has a direct stimulatory effect as well and may thus play a role in the pathogenesis of alcohol-induced liver fibrosis.

XII Effect of Dimethyl Sulfoxide and Glycerol on Acute Bowel Ischemia in the Rat

Despite significant advances in diagnosis and treatment, intestinal ischemia due to thromboembolism and low flow states remains a challenging clinical problem.

Hydroxyl radicals are believed to play a significant role in the pathogenesis of tissue ischemia (3). During the ischemic period, free radicals can be formed by peroxidation of cellular membrane-bound lipids (4). Dimethyl sulfoxide and glycerol are known to be effective free radical scavengers (5, 6). Both compounds have also been shown to impair platelet aggregation (6) In view of these observations, we carried out a series of experiments to determine whether dimethyl sulfoxide and glycerol have a cytoprotective effect when given to rats with experimentally induced acute intestinal ischemia.

Adult male Sprague-Dawley rats weighing 250-300 g were used. All animals were anesthetized with pentobarbital sodium (3 mg/100 g intraperitoneally). Ether was used to supplement anesthesia as needed. The abdomen was clipped and cleansed with alcohol, and a 2.5 cm midline abdominal incision was made. The superior mesenteric artery was exposed close to its origin from the aorta and occluded for 30 minutes with an atraumatic vascular clamp. The right femoral vein was identified in the groin through a separate incision, and it was cannulated using a 24 gauge catheter. At the end of the ischemic period, the vascular clamp was removed and blood supply to the intestina was reestablished. The superior mesenteric artery pulse was present in all the animals after removal of the clamp. Both the abdominal and groin incisions were closed using continuous 3-0 silk sutures.

Immediately after removal of the superior mesenteric artery clamp, Group 1 rats(n=20) received 8 ml of pyrogen-free sterile 0.9 percent sodium chloride into the right femoral vein at a constant rate (1 ml/min) using an infusion pump. Group 2 rats(n=20) received a 20 percent glycerol in 0.9 percent sodium chloride infusion at the same rate and volume. Group 3 rats (n=20) received 20 percent dimethyl sulfoxide in 0.9 percent sodium chloride infusion at the same rate and volume. The infusion catheter was removed at the end of the infusion period, and the vein was ligated. On the second postoperative day, all animals were killed by ether overdosage, the abdomen was explored, and the appearance of the peritoneal cavity was examined and graded as described herein by two observers independently in a blind code fashion. Segments of intestine were resected at the end of the experiment for histologic examination.

In another series of experiments, three groups of rats were given 0.9 percent sodium chloride (n=20), 20 percent glycerol in 0.9 percent sodium chloride (n=20), or 20 percent dimethyl sulfoxide in 0.9 percent sodium chloride (n=20) intraperitoneally in 4 ml dosages after removal of the superior mesenteric artery clamp. All animals were killed with ether on the second postoperative day and graded by two observers independently as described herein in a blind code fashion.

The peritoneal cavity was assessed as to the presence or absence of the following: free peritoneal fluid, peritoneal adhesions, areas of patchy intestinal gangrene, ileus, and, intraabdominal hemorrhage. Each of these findings was assigned a grade of 0 if absent, of 1 if present. If all five were present, the grade was 5. Thus, the grade varied from 0 to 5 depending on how many abnormal findings were present. Areas of bowel necrosis were confirmed by histologic examination.

None of the rats that received either intravenous or intraperitoneal saline solution, glycerol, or dimethyl sulfoxide infusion died after superior mesenteric artery occlusion. All animals were killed at 48 hours. All rats that received the intravenous normal saline infusion demonstrated severe inflammatory reaction, presence of hemorrhagic fluid in the peritoneal cavity, extensive adhesion formation, severe ileus, and areas of bowel wall necrosis. The mean grade in the normal saline group was 5 (Table 14). Rats that received the 20 percent glycerol infusion also exhibited

ileus and hemorrhagic fluid in the peritoneal cavity but minimal adhesion formation and a moderately severe inflammatory reaction. The mean grade in this group was 4.1, which was not significantly different from that of the saline control group (Table 14). Rats that received the 20 percent dimethyl sulfoxide infusion had essentially a normal-appearing peritoneal cavity, and the only two abnormal findings seen were a mild ileus and the presence of small amounts of free peritoneal fluid. The mean grade in this group was 1.4, which is significantly lower than that of the saline control group (Table 14). Table 14 also shows that intraperitoneal administration of either glycerol or dimethyl sulfoxide had no protective effect in this model of experimental intestinal ischemia.

Our experimental results using a reproducible model of acute intestinal ischemia demonstrate that dimethyl sulfoxide has a dramatic protective effect when given to rats intravenously at the end of the ischemic period. Glycerol was not found to be effective in this model. Neither agent was effective when given intraperitoneally. This is difficult to explain because dimethyl sulfoxide is rapidly absorbed across body membranes. However, it is possible that intraperitoneal dimethyl sulfoxide or glycerol may cause intravascular volume contraction by being hyperosmolar, resulting in third-space fluid sequestration into the peritoneal cavity. Such an effect may result in further impairment of flow through the mesenteric circulation. This postulated mechanism could also be used to explain the beneficial effect of dimethyl sulfoxide. Intravenous dimethyl sulfoxide acts as a plasma expander, and it may result in increased intravascular volume, increased cardiac output, and improved perfusion of the mesenteric circulation.

Although the actual mechanism of the dimethyl sulfoxide effect in this system is not known, the combination of the general antiinflammatory effect, membrane stabilizing effect, free radical scavenger action, platelet antiaggregant effect, plasma expander effect, or each one of these actions alone could explain the effectiveness of dimethyl sulfoxide as a cytoprotective agent in this model of acute experimental intestinal ischemia. The striking results of this study, which confirm other studies (7) suggest that further investigation is needed to elucidate the mechanism of this effect and to apply this therapeutic regimen to the clinical setting.

Table 14

Grading of Peritoneal Cavity Appearance in Rats With Superior Mesenteric Artery Occlusion 30 Minutes After Administration of Various Agents*

Infusion Group	Intravenous Administration		Intraperitoneal Administration	
	Grade	p Value	Grade	p Value
Normal saline solution	5 ± 0	...	5 ± 0	...
Glycerol	4.1 ± 1.5	NS	4.2 ± 1.1	NS
Dimethyl sulfoxide	1.2 ± 0.8	<0.01	4.4 ± 1.5	NS

* Grades are indicated as the mean grade ± standard error of the mean.

NS = not significant.

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Effect of Dimethyl Sulfoxide and Glycerol on Acute Bowel Ischemia in the Rat

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XIII Acquired Local Resistance to Burns: Prevention of Its Acquisition by Chlorpromazine

Background and Rationale:

We have been interested for some time in the possibility of minimizing the local and systemic effects of trauma by the induction of local resistance to trauma and suggested a number of years ago that it might be possible to "immunize" against certain types of trauma by prior, controlled exposure.

The phenomenon of acquired resistance to trauma has been recognized since the Noble-Collip drum studies of the 1940's (Noble, R.L., Am. J. Physiol. 138:346, 1942) in which rats subjected to graded amounts of trauma were shown to survive a level of trauma fatal for normal rats. Ungar, G. (Lancet 1:421, 1943) extended this concept to show that the phenomenon occurred with other types of injury. In his experiments, two types of trauma were used; one, the shooting of a steel projectile through the adductor region of a thigh of a guinea-pig to create an open wound, and the other, the dropping of a steel rod on the thigh of a rat in an attempt to mimic closed blunt trauma. Untreated survivors from the initial trauma had increased survival after more severe subsequent trauma, that is, an increased velocity of the steel projectile or a greater crushing weight. Rosenthal and Tabor (Am. J. Physiol., 143: 402, 1945) demonstrated that acquired resistance could be achieved on a local tissue level using repetitive tourniquet injury to the limbs of mice. Hoene (Proc. Soc. Exp. Biol. Med., 85: 56, 1954), in a related study, utilized burns to show that pre-injury of a rat paw by heat locally protected that area against a subsequent burn without affecting the usual inflammatory response to burning of other areas. Selye (Science 156: 1262, 1967; Biochem. Pharmacol. (Suppl.) 17: 197, 1968) examined the effects of various drugs and stressors on the severity of ischemic necrosis produced by the 9-hour application of a dorsal skin clip to rats. A tenfold decrease in the amount of ischemic necrosis was attained by pretreating the animals 30 minutes prior to injury with the drug chlorpromazine administered subcutaneously at a dosage of 1.5 mg per 100 g body weight.

Hypotheses

Because chlorpromazine minimized the injurious effects of ischemia in Selye's experiments, we hypothesized that it would likely do the same in burn injury. We also hypothesized that chlorpromazine, by minimizing the effects of the initial burn injury, would prevent the acquisition of local resistance to subsequent burn injury.

Experiments

Male Sprague-Dawley rats, 100-120 g in weight, were fed a standard rat chow and given tap water ad libitum throughout the experiment. They were divided into two groups of 11 animals each, paired by similar weights. The first group served as controls, while the second group received 1.5 mg of chlorpromazine subcutaneously 30 minutes prior to injury. All rats were burned under pentobarbital anesthesia by a method similar to that described by Hoene (Proc. Soc. Exp. Biol. Med., 85: 56, 1954). Specifically, their right hind paw was circumferentially marked at the ankle, and then placed into 48.5°C water up to this mark for 2 min. 15 sec., creating a partial thickness scald burn; there was no hair loss or slough of the burned skin at any time. The burn oedema was measured using a plethysmograph. The volumes of the rat paws were measured immediately prior to burning, hourly for the first 7 hours and at 24 hours postburn.

Three days following the initial burn, both hind paws of all rats were burned in the manner already described, but at a slightly higher temperature and longer time period (49.5°C, and for 2 min. 30 sec.). No chlorpromazine was administered to any rat at this time. Volumes of both paws of all animals were again measured plethysmographically at hoursly intervals for the first 7 hours, and at 24 hours post injury.

The data obtained were analysed statistically using the paired Student's t test for comparison of opposite paws of the same animals, and the unpaired Student's t test for comparison of like paws of the control and chlorpromazine groups. Because multiple results over varying time intervals were obtained in these experiments, we adjusted the significance levels by the Bonferroni procedure, a method which gives more conservative probability levels (Abt, K., Controlled Clinical Trials, 1: 377, 1981).

The paws of control rats showed visible and marked swelling after the first burn when compared to the unburned paw and when compared to the burned paw of the chlorpromazine treated animals. The oedema in the burned paws of the control rats developed within one hour, peaked at three hours, and then decreased progressively during the next four hours. The paws returned to their pre-burn size in about seven hours. In contrast, the burned paws of the chlorpromazine treated group developed drastically less oedema (a factor of 7, $P < 0.001$) at the peak (three hours) and the decrease in oedema was somewhat faster, the paws returning to their pre-burn size in about 6 hours.

Three days later, both hind paws of each rat were burned. The control rats' left hind paws, which had not been previously burned, showed a more rapid onset of oedema and considerably greater levels (a factor of 7, $P < 0.001$) at the peak (3 hours after burn) than the reburned right hind paw. The oedema of both paws disappeared within 24 hours post-injury. These data demonstrate that the previously burned paw had acquired local tissue resistance to the subsequent burn injury.

In contrast, in those rats treated with chlorpromazine three days earlier, both burned paws (the right hind paw which had been burned previously and the left hind paw now burned for the first time) showed similar marked oedema following reburn. In this case, peak oedemain both paws occurred three hours post-injury, and disappeared within 24 hours. This demonstrated that the acquisition of local resistance to subsequent burn by previous burning was almost completely prevented in this group.

In summary, we found the following: (1) recovery from a previous partial thickness scald burn induced local resistance to subsequent burn injury; (2) chlorpromazine administered 30 min. before burning dramatically minimized burn injury, lessening the peak oedema sevenfold; and (3) the acquisition of local resistance to injury induced by a previous burn was almost completely prevented by chlorpromazine.

We have discussed our views of chlorpromazine's actions underlying these findings in detail in our published paper (Bibi, Babyatsky, Levenson, Burns 9: 387 (1983)). We plan to extend these studies to large area burns to determine the effect of prior burn on the effects of subsequent burn, e.g., degree of skin damage, plasma volume changes, fluid and electrolyte requirements, shock, and mortalidy, including the development of local resistance to extensive thermal injury. The effects of chlorpromazine (prophylactically and therapeutically, on these various factors will be assessed also.

These experiments dealing with acquired local resistance to injury have, we believe, important clinical implications.

XIV Supplemental Dietary Tyrosine in Hemorrhagic Shock and Sepsis

Shock and sepsis are two frequent and major complications of seriously injured and ill patients. Dopamine (DA) and Norepinephrine (NE) play important protective roles in the body's response to stress, including shock and sepsis. For example, DA and NE levels are reduced in rat brain following stress (e.g., cold swim, electrical shock) with a concomitant increase in the levels of their metabolites. Tyrosine (TYR), an amino acid found in proteins is the precursor of DA and NE. Rats fed supplemental tyrosine have higher brain levels of TYR and DA but not NE and when stressed show increased behavioral activity (aggression, exploration). Accordingly, we hypothesized that increased catecholaminergic activity in TYR fed rats as a result of alteration in DA and NE metabolism would enhance resistance to hemorrhagic shock and sepsis. The following experiments were carried out to test this hypothesis:

Exp. 1: Adult male Sprague-Dawley rats (n=22) were divided into two dietary groups: a standard laboratory chow (234 g protein/kg chow containing 6.8 g TYR/kg chow) or the same chow supplemented with L-TYR (10g/kg chow). Four days later, rats from each group underwent cannulation of both femoral arteries, under i.p. pentobarbital anesthesia, for continuous blood pressure (BP) monitoring and blood withdrawals. After BP equilibration, 1 ml of blood was removed and the BP recorded at 2, 5, and 10 minutes. After the 10 min. recording, another 1 ml was removed and the cycle repeated until cardiac arrest. Percent blood volume (BV) removed was calculated by dividing the total BV removed by the estimated total BV (6% of BW). There was a significant increase in % BV removed in TYR supplemented rats, 54.2% vs 40.6% ($p < 0.01$) in control rats.

Exp. 2: Adult male Sprague-Dawley rats (n=36) were divided into two dietary groups as in Exp. 1. After seven days on their respective diets, the rats underwent cecal ligation and perforation of the ischemia ligated cecum with a #18 gauge needle under i.p. pentobarbital anesthesia. There was a significant ($p < 0.02$) increase in rat survival (14 days) in the TYR supplemented rats (13/18, 72.2%) when compared to controls (5/18, 27.7%).

In conclusion, we have shown that rats prefed supplemental TYR are better able to tolerate acute fulminant hemorrhagic shock and sepsis. These findings have important implications for the formulation of optimal diets, both intravenously and oral, for seriously ill and injured patients.

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