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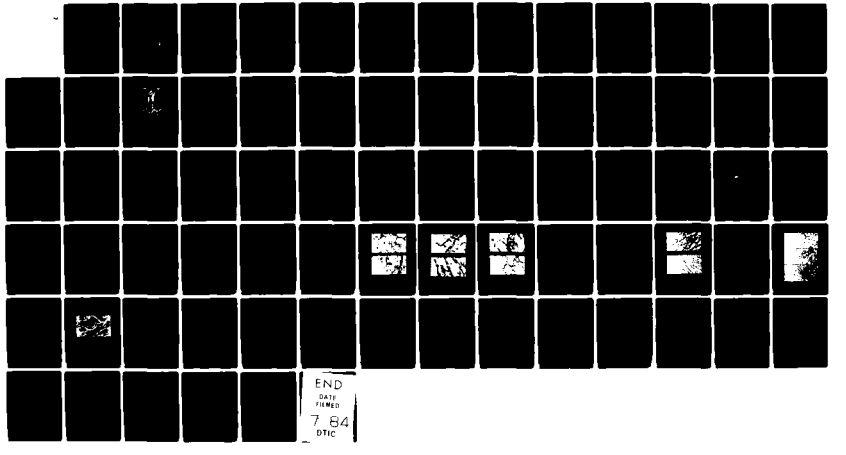

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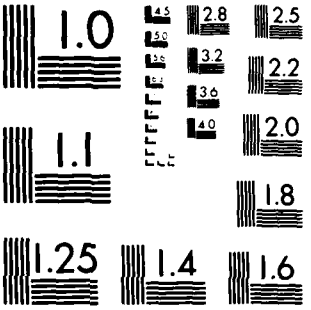

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This work involves the developement and testing of a new crosslinking method used in preparation of a collagen-based dermal equivalent. In addition to this, the sponge surface characteristics and the control of these characteristics has been studied. This new dermal equivalent is more fibrous and open meshed, and is more like the in vivo extracellular matrix than those reported in the literature.

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Sponge topography was studied after variation of pH and freezing conditions. The results of these studies indicate that: 1) the optimum pH range for uniform fibrous microstructure was between 3.5 and 3.75 and 2) the most uniform sponges were obtained after freezing in an ethanol bath at -55° C.

Sponges prepared under these conditions are similar in their morphology to the well known fibrous scaffold that reinforces all mammalian extracellular matrices. These sponges are useful as a temporary biodegradeable dressing to enhance wound healing between the time of mechanical or thermal injury and the time that autografting is possible.

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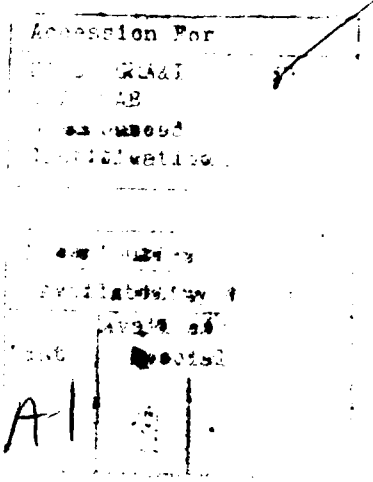

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By CONRAD FRANCIS WHYNE

A thesis submitted to
The Graduate School-New Brunswick
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for the degree of
Master of Science
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Written under the direction of
Professor Frederick H. Silver
and approved by

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ABSTRACT OF THE THESIS

Evaluation of Crosslinking Methods and
Characterization of Surface Features of a
Collagen-Based Dermal Equivalent

by CONRAD F. WHYNE, B.S.

Thesis Director: Professor Frederick H. Silver

This work involves the developement and testing of a new crosslinking method used in preparation of a collagen-based dermal equivalent. In addition to this, the sponge surface characteristics and the control of these characteristics has been studied. This new dermal equivalent is more fibrous and open meshed, and is more like the in vivo extracellular matrix than those reported in the literature.

Three different crosslinking procedures were studied that involved succinimidyl ester formation. One of the procedures, where collagen was exposed to succinic anhydride, N-hydroxysuccinimide and cyanamide prior to sponge formation, resulted in the longest collagenase resistance time and had the highest value of the volume fraction of polymer.

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1. INTRODUCTION

Skin is one of the most important organs of our body. Its importance is highlighted by the many problems that confront the severe burn patient. These patients lack sensation of pain, heat and cold because the cutaneous nerves are destroyed. After skin destruction, microorganisms and chemicals penetrate through the burn to deeper tissues causing infections and toxic complications. Loss of proteins and ionic fluids through the damaged areas create electrolyte imbalance and severe dehydration. In the absence of the cutaneous vascular system, the burn patient is unable to conserve or dissipate heat adequately (Stenn, 1983). This thesis extends the large amount of research being conducted in the area of artificial skin by introduction of a new crosslinking procedure. Materials crosslinked with this technique are characterized by scanning electron microscopy. The scope of this thesis also involves the factors that affect the nature of the collagen sponge surface characteristics.

From a physiological standpoint, the skin serves as a barrier and regulator between the body and the unpredictable environment. In addition however, this function must be performed in ways that will permit maximum mobility, adaptability and survivability of the organism it covers (Bauer and Uitto, 1982).

The skin is a stratified tissue consisting of two major layers. The outermost layer is the epidermis and below this is the dermis. The epidermis is essentially cellular material, whereas the dermis is primarily a fibrous structure usually much thicker than the epidermis

(Romer and Parsons, 1977).

The epidermis consists of the following cell layers: the stratum corneum, stratum lucidum, stratum granulosum and statum malpighii. The dermis has the papillary layer and the reticular layer (Figure 1.1). Within the dermal organization of the skin, collagen is 70% of the dry weight of a typical sample. Thus the biological function of collagen is to provide a structural framework and through interactions with other tissue macromolecules such as glycosaminoglycans, proteoglycans, and elastin, to provide the critical structural integrity necessary for the skin to perform its primary functions (Bauer and Uitto, 1982).

One of the most serious injuries to skin is a burn. The National Burn Information Exchange has estimated that about 2,000,000 Americans (about 1 in 10 of the population) are burned each year. Approximately 70,000 require hospitalization and more than 9,000 die (Feller, 1980). Since the skin is a barrier to fluid loss and bacterial infection, a burn, depending on severity, that breaks this barrier is a serious injury which must be dealt with swiftly and aggressively.

The areas damaged by a burn must be repaired. This repair is a dynamic process involving cellular migration and proliferation. Fibroblasts and endothelial cells migrate from the wound edge and form a newly vascularized connective tissue network. This directed migration, a process by which extracellular substances determine the direction of cell mobility, must be one of the prime controlling mechanisms of wound repair.

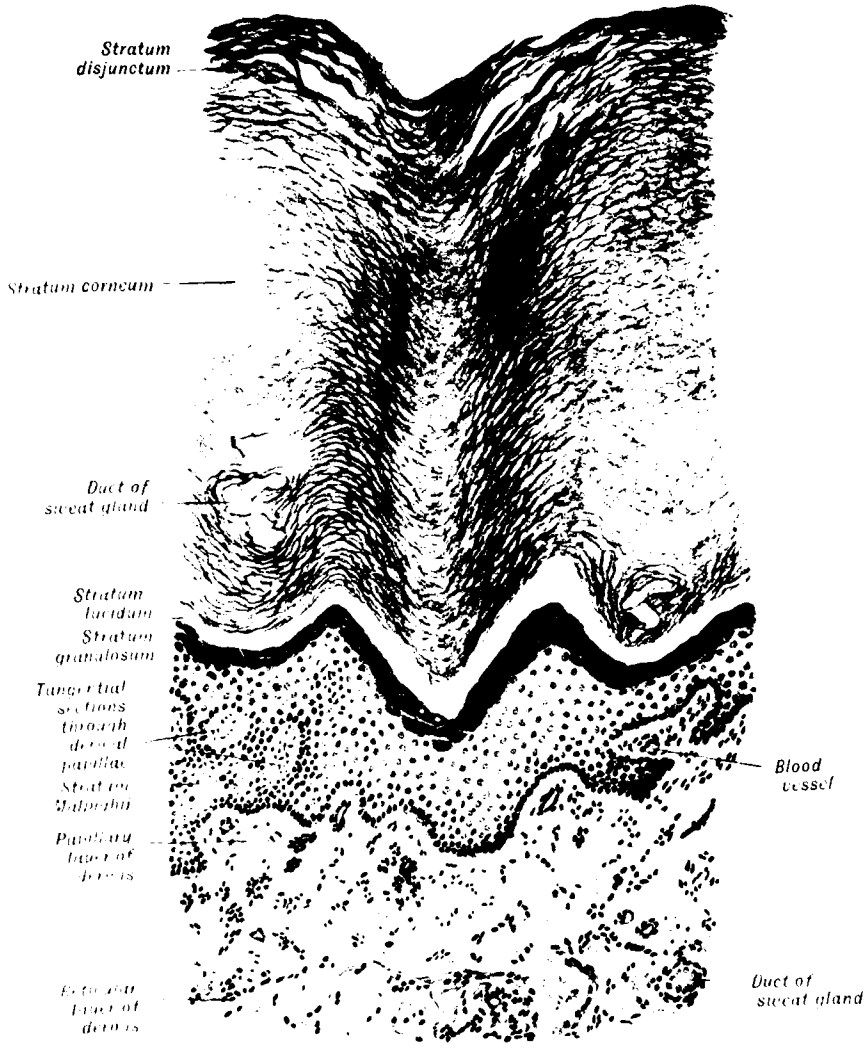


Figure 1.1 Cross section of human skin. From Bloom and Fawcett, 1975.

During normal wound repair, the intitial injuries are followed by an inflammatory response. The inflammatory response is characterized as a vascular and cellular response designed to support the body in its defense against foreign material and to rid the body of dead and dying tissue in preparation for the repair process (Peacock and Van Winkle, 1976).

The vascular response is, to a large extent, dependent on the actions of several mediators. This response starts when the damaged tissue releases chemical mediators such as histamine and serotonin (Davis et al., 1981), which increase local microcirculation permeability. Cell metabolism of arachidonic acid produces prostaglandins, leukotienes and other mediators (Parker, 1982). Prostaglandin E_1 antagonizes vasoconstriction, thus increasing vascular permeability (for a review see Peacock and Van Winkle, 1976). Leukotrienes are potent stimulators of vascular permeability especially in the presence of prostaglandin E_2 (PGE_2) (Bray, 1982). PGE_2 also enhances the edema producing properties of bradykinin (Parnham and Winkelmann, 1982). Other mediators such as platelet-derived growth factor are chemotactic to fibroblasts and cause their proliferation (Seppa et al., 1982). These mediators donot only act independently but act synergistically to bring about the cellular response.

The cellular response consists of an influx of neutrophilic granulocytes, macrophages, lymphocytes and fibroblasts. Repair by these cells commences almost immediately. The macrophages are phagocytic, neutrophils release hydrolytic enzymes, lymphocytes fight

infections and fibroblasts proliferate and produce collagen and other connective tissue components. Epithelial cells migrate onto the granulating tissue, and as these cells proliferate, collagenase is liberated, paving the way for subsequent wound remodeling (Peacock and Van Winkle, 1976).

Types of burns in the skin range from 1st degree burns involving only the epidermis to 4th degree burns that go down to the bone (Table 1.1).

Table 1.1 The classifications of burn and the extent of tissue damage. Derived from Rudowski, et al., 1976.

CLINICAL CONDITION	ANATOMICAL CHANGES	DEGREE CLASSIFICATION
erythema	necrosis of epidermal layer	I
blisters		IIa
partial skin necrosis	superficial necrosis of dermal layer, deepest layer preserved	IIb
full skin necrosis	necrosis of full dermal layer	III
necrosis of deeper organs	necrosis of deeper organs, with anatomical changes	IV

In order for wound repair to take place, the deeply burned patient's open wound must be converted into a closed one. This conversion effectively prevents toxic and infectious complications (Oluwasanmi et al., 1976). This conversion requires a temporary or permanent wound dressing or covering. The most quoted criteria for a suitable covering or dressing are found in Table 1.2.

Table 1.2 A compilation of the typical criteria cited for a burn wound covering or dressing

Criteria for a suitable wound covering or dressing.

1. Easy application (Yannas and Burke, 1980)
2. Adherance to wound surface (Yannas and Burke, 1980, and Oluwasanmi et al., 1976)
3. Conformability (which includes suppleness, resiliency and ability to mimic wound topography) (Yannas and Burke, 1980)
4. Prevention of fluid loss (Yannas and Burke, 1980)
5. Prevention of heat loss (Oluwasanmi et al., 1976)
6. Protection against mechanical injury (Oluwasanmi et al., 1976)
7. Elimination of secondary bacterial contamination (Oluwasanmi et al., 1976)

In addition, the foreign material must allow normal wound contraction, but prevent contracture (abnormal contraction) from occurring, be nontoxic, nonantigenic, stable and suppress any infections already present (Norton et al., 1981). All of these properties are unlikely to be found in any single material.

Common wound coverings used today are: 1)autografts - skin harvested from unburned areas of the patient, 2)allografts - human skin from donors or cadavers and 3)xenografts (heterografts) - porcine skin which must be replaced every 3-4 days before adhesion and rejection occurs. Allografts and xenografts are effective for only a short period of time before rejection occurs, again leaving the wound open (Peacock and Van Winkle, 1976).

However, when the ideal covering, the autograft, is not available because of insufficient donor skin area (due to large burns) researchers have looked to an artificial skin to provide a temporary or permanent covering.

Collagen is presently the material of choice to make this artificial skin. Membranes made from reconstituted collagen have been used to treat burns (Guldalian et al., 1973); however, none have been widely accepted. For the past 14 years, the team of Yannas and Burke and their coworkers have collaborated on an artificial skin using reconstituted collagen in the form of a glutaraldehyde crosslinked bilayer sponge with the top being a silicone rubber to prevent fluid loss (Yannas, 1981).

Why is collagen a logical choice for a biomaterial? The physical-mechanical, physical-chemical, and biological properties of

collagen are what make it a promising biomaterial (Table 1.3).

TABLE 1.3 Factors favoring the use of collagen as a biomaterial in wound dressings. From Chvapil, Kronenthal, and Van Winkle, 1973.

1. PHYSICAL-MECHANICAL	High tensile strength Low extensibility Orientation of fibers
2. PHYSICAL-CHEMICAL	Controllable cross-linking by tanning agents; affects soluability, swelling, resorption Ion exchanger function Semipermeability of membranes
3. BIOLOGICAL	Low antigenicity Effect on wound healing Effect on blood coagulation

The use of collagen as a biological dressing offers several advantages. Collagen, because of its abundance in mature bovine hides, can readily be isolated and purified in large amounts. Its structure and immunological chemistry are already characterized. It is weakly antigenic in its pure state and possesses a good hemostatic effect on coagulation (Tavis, 1975). Also of importance is its ability to be made into a variety of physical structures (sheets, sponges and powders). All these advantages support the use of collagen as a biological material.

There are several types of collagen that have been characterized as illustrated by Table 1.4. Type I collagen is the most common type found in the skin as well as other tissues. Collagen is a rod-like molecule approximately 3000 A long and 14 A in diameter consisting of a triple helix composed of three polypeptide chains each of which has a molecular weight of approximately 95,000 daltons (Glanville and Kuhn, 1979). The central region of each chain is composed of 1,014 amino acid residues with a repeat pattern of gly - x - y (Prockop et al., 1979, and Brodsky et al., 1982). The x and y positions are usually filled by proline and hydroxyproline, respectively. These amino acids help to direct the helix formation of the alpha chains by virtue of rotational restrictions. Each of the three alpha chains is a left hand helix, while the center of the collagen molecule forms a right hand triple helix (Glanville and Kuhn, 1979). Outside the center triple helix section are the terminal peptide regions known as the telopeptide segments. These segments do not intertwine to form a triple helix. The amino terminal segment of the alpha 1 type I chain

Collagen type	*Molecular configuration*	*Tissue or organ source**
Interstitial		
I	$(\alpha 1(I))_2\alpha 2(I)$	Skin, bone, tendon, cornea, annulus fibrosus, placental tissue, lung, liver, muscle
I trimer	$(\alpha 1(I))_3$	Skin, tumour, tendon, liver
II	$(\alpha 1(II))_3$	Cartilage, annulus fibrosus, nucleus pulposus, vitreous body
III	$(\alpha 1(III))_3$	Fetal skin, aorta, uterus, placental tissues, synovia, heart, liver, lung, nerve
V	$(\alpha 1(V))_2\alpha 2(V)$	Placental tissues, skin, bone, tendon, synovia, cornea, aorta, nerve, lung, liver, muscle
	$(\alpha 1(V)\alpha 2(V)\alpha 3(V))$	Placental villi and uterus
Basement membrane		
IV	$\alpha 1(IV)$ and $\alpha 2(IV)$ or possibly $(\alpha 1(IV))_3$	Lens capsule, glomerulus, placenta, tumour, aorta Descemet's membrane
Other collagens		
1α, 2α, 3α	unknown	Cartilage, annulus fibrosus, nucleus pulposus
High molecular weight aggregate	unknown	Placental tissues, skin, liver, uterus
7-S	unknown	Lens capsule, tumour, placenta
CPS-1	$(33K)_3$-part of larger molecule?	Cartilage, annulus fibrosus, nucleus pulposus, vitreous

TABLE 1.4 The distribution and molecular classification of different known collagen types. From Weiss and Ayad, 1982.

is 16-17 amino acid residues long and amino terminus of the alpha 2 type I chain is 9-11 residues in length. The opposite end, the carboxyl terminal segment of the alpha 1 type I chain has a length of 25 residues, while the alpha 2 type I segment has 6-8 residues (Fraser et al., 1979)

Crosslinking occurs in these telopeptide regions. It is the formation of crosslinks that stabilize the molecular arrangement within the fibrils and provide collagen fibers their resistance to chemical attack (Bailey et al., 1974, and Linsenmayer, 1981).

Previous studies have shown the importance of crosslink density of collagen sponges on degradation and wound healing. Weadock (1983) demonstrated that increased crosslinking decreases the enzymatic degradation in vitro. Both Chvapil et al. (1977) and White (1973) have shown that increased crosslinking with glutaraldehyde delayed the in vivo resorption of collagen implants. However glutaraldehyde has been linked to cytotoxicity (Speer et al., 1980, and Cook et al., 1983) among other problems (see discussion). So new methods of crosslinking have been researched. Weadock (1983) has shown a combination of dehydrothermal crosslinking and exposure to a cyanamide solution to be very effective in crosslinking reconstituted collagen sponge. This thesis explores another crosslinking method using succinic anhydride to acylate collagen, then through the use of a N-hydroxysuccinimide and cyanamide solution, conversion of the acylation group to an active ester which spontaneously reacts with free amine groups to form intramolecular crosslinks.

However, crosslinks are not the only important consideration when

fabricating an artificial skin. Elsdale and Bard (1972) and others have shown that collagen substrates in the form of hydrated collagen gels are the best material for cell culture in vitro. Cells retain their natural in vivo shape and motility and continue to spread in this environment (Grinnel and Bennet, 1982 and Elsdale and Bard, 1972). However, cells grown on glass, plastic or collagen sheets (air dried) do not retain their natural in vivo shape because the cells require a 3-dimensional substrate in which to thrive naturally (Grinnel and Bennet, 1982). So porousity, which allows cell ingrowth is another important consideration in making an artificial skin. A porous collagen matrix is obtained by dispersing the collagen in an acid solution, freezing the dispersion and then freeze-drying the dispersion to form a sponge. An additional advantage to using a collagen sponge is that it controls wound fluids better than a non-porous structure could (Chvapil, 1982).

These recent advances in cell culture have shown the strong influence the substratum has on the individual cells in vitro (Fisher and Solrush, 1979 and others). So along with a new crosslinking procedure, a scanning electron microscope study was done to determine the factors influencing and conditions which affect pore size and matrix configuration. Once these factors and conditions are established, methods of controlling them could be utilized to make reconstituted collagen sponges more conducive to rapid cell ingrowth and proliferation. The factors examined in this thesis were pH, rate of freezing (based on initial temperature into which the collagen dispersion was placed) and the freezing environment.

2. MATERIALS AND METHODS

The collagen used in these experiments was donated by Devro, Inc, Somerville, N.J. The collagen was extracted from fresh, uncured bovine hides. The collagen was prepared from the corium of mature bovine hides in a process similar to that process described by Komnowsky et al. (1974). The hides were limed, dehaired, split, chopped into small pieces and then swollen with acid. The collagen (BHC) was precipitated from aqueous solution and then lyophilized. The collagen was received from Devro in this condition and stored at -30^{o} C until it was used. Weadock (1983) characterized this collagen by SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis. It was characterized as typical of type I collagen without noncollagenous protein contamination. An amino acid assay by the Connective Tissue Laboratory (University City Science Center, Philadelphia, Pa.) was performed, the results are in Table 2.1. The results fit very well for what is expected of animal type I collagen.

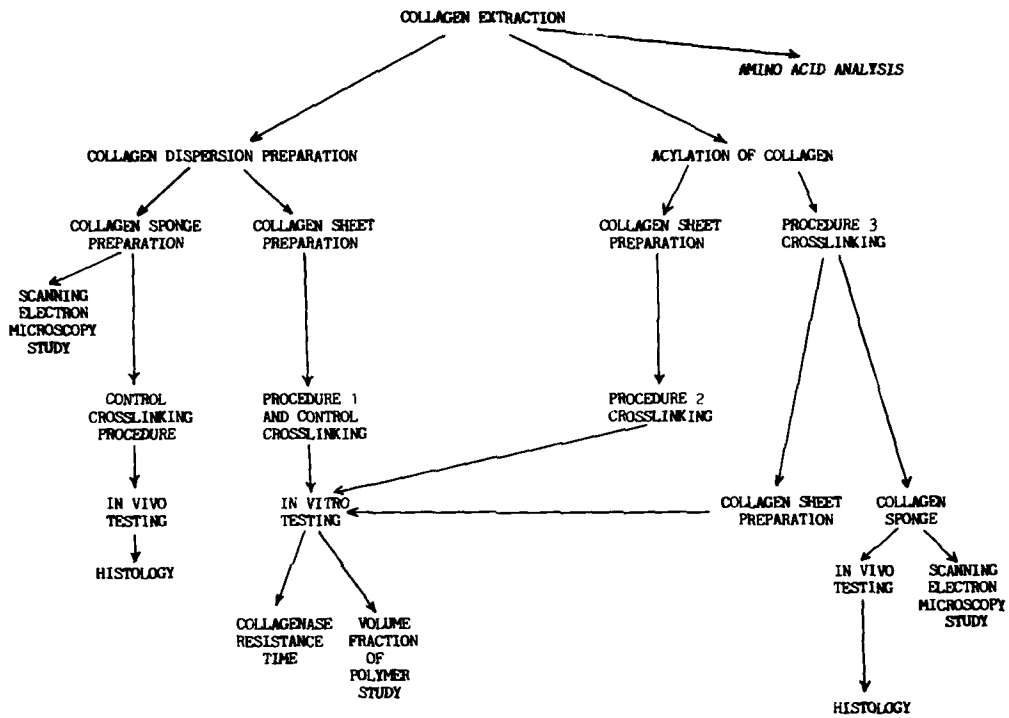
Figure 2.1 outlines the general protocol used in this thesis. Only the procedure that created the most tightly crosslinked material was evaluated in the animal studies (a full thickness graft onto the panniculus carnosa on the back of guinea pigs).

In order to evaluate the effectiveness of the three crosslinking procedures, a collagenase resistance time and a volume fraction of polymer test were performed. The controls used in these experiments were uncrosslinked 1% w/v (weight of collagen to volume of pH 2.0 HCl

TABLE 2.1 Amino acid composition of collagen obtained from mature bovine skin and used in these experiments. The known composition of bovine type I and type III collagen are shown for a comparision. The analysis and the known compositions are given as the number of residues of the amino acids present per 1,000 residues. The analysis indicates that the sample is primarily type I collagen. From Kuhn, 1982.

RESIDUE	RESIDUES PER 1,000 IN SAMPLE	TYPE I RESIDUES PER 1000	TYPE III RESIDUES PER 1000
4 HYP	88	86	120
ASP	45	46	47
THR	17	16	16
SER	33	31	48
GLU	73	75	71
PRO	123	130	106
GLY	319	327	341
ALA	128	114	95
VAL	27	23	16
METH	5	6	8
ILEU	11	12	14
LEU	24	25	18
TYR	3.4	3.6	4
PHE	11	13	9
HYL	6	7	7
LYS	24	28	28
HIS	5	5	7
ARG	54	52	45
CYS	0	0	2

Figure 2.1 Outline of general protocol



solution) of bovine hide collagen dispersion and a combination of dehydrothermal/cyanamide crosslinking as outlined by Weadock (1983).

In order to evaluate the in vivo response, materials crosslinked by Procedure 3 (P3) and the P3 dehydrothermal (P3DHT3) combination were grafted onto full thickness excisions on the back of Hartley albino female guinea pigs (Perfection breeders, Douglasville, Pa.). Also 1% w/w fibronectin (based on collagen weight, extracted from bovine plasma by Dr. Charles Doillon), 5% hyaluronic acid (HA) (potassium salt, grade III-p, from human umbilical cord, Sigma Chemical Co., St Louis, Mo.) and a 1% fibronectin/5% HA combination were incorporated into the P3 and P3DHT3 sponges. Sponges are made from 60 mls of a 1% collagen acid dispersion (weight of collagen to volume of pH 2.0 HCl solution) which are then frozen and freeze-dried. The controls used in these experiments were uncrosslinked 1% BHC sponges, DHT3 1% sponges (Weadock, 1983) and C1DHT3 1% sponges (Weadock, 1983). The in vivo responses were analysed based on histological events.

2.1 COLLAGEN DISPERSION PREPARATION

A 1 N solution of HCl was slowly added to 180 mls of distilled water in a 400 ml beaker until the pH was 2.0, using a model I 43 Beckman pH meter (Beckman Instrument Inc., Fullerton, Ca.). The contents of the beaker were emptied into a graduated cylinder, to which distilled water was added to make 200 mls of solution.

A 2.0 gm sample of collagen, obtained from Devro Inc, was weighed out on a balance (Model 1212 MP Sartorius, Sartorius GMBH, Gottingen,

W. Germany). The 2.0 gms of collagen and the 200 mls of the pH 2.0 HCl solution were placed in a blender (Osterizer Blender, Galaxie dual speed model, Sunbeam Corp, Milwaukee, Wis.). This 1% w/v collagen/acid dispersion was blended at high speed for 2 minutes.

The dispersion was then emptied from the blender into a 1000 ml sidearm flask. A vacuum (Vacuum pump, model 150, Precision Scientific Co., Chicago Ill.) of 300 microns (Thermocouple Vacuum Gauge, Type GTC-100, Consolidated Vacuum Corp., Rochester, N.Y.) was then applied at room temperature until the air bubbles were removed from the dispersion. This procedure required approximately 10 minutes. The vacuum was then removed, and the dispersion was now ready for sheet or sponge preparation.

2.2 COLLAGEN SHEET PREPARATION

Sheets were prepared by taking 30 mls of the dispersion prepared in Section 2.1 and pouring it into plastic trays (12.5 cm x 8.5 cm x 1 cm) refered to as "boats." The trays were loosely covered with aluminum foil to prevent entry of foreign material and allowed to dry at room temperature for 36-48 hours. The collagen sheets were removed from the trays with forceps and placed in sealed plastic bags for storage until use.

2.3 COLLAGEN SPONGE PREPARATION

For sponge preparation, 60 mls of the dispersion prepared in Section 2.1 were poured into plastic trays (12.5 cm x 8.5 cm x 1 cm). The dispersion was spread evenly within the boats.

For the scanning electron microscope study, sponges were made at various pH dispersions. The dispersions had the following pHs: 2.0, 2.25, 2.5, 2.75, 3.0, 3.25, 3.5, and 3.75 (the pH was determined after the dispersion had been blended). Sponges of these pH's were made of both uncrosslinked and crosslinked collagen. In addition, the freezing conditions under which the sponges were frozen was varied. The sponges were frozen by placing the boats in a bath of 1200 mls of cooled 95% ethanol. The boats were allowed to float in the bath until frozen. Each sponge was frozen individually. The temperature was varied from -90° C to -30° C at 10° C intervals. After the initial experiments, only -80° C, -55° C and -30° C were used. When an ethanol bath was not used to freeze the sponges, a metal plate was placed on a freezer shelf which was maintained at -30° C.

The frozen dispersion was then placed in the specimen chamber of a freeze-drier (Freeze-Mobile 12, Virtis, Inc., Gardner, N.Y.) at room temperature. A vacuum of 10 microns was then applied until the sponge was completely lyophilized. The vacuum was then released and the samples removed. The collagen sponges were removed from the boats and placed in sealed plastic bags for storage purposes.

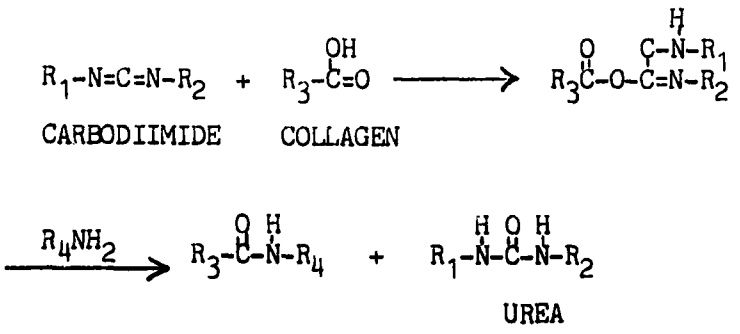
For in vivo experiments, the lyophilized sponges were coated with a thin superficial layer of silicone rubber (Silastic, Dow Corning, Corning, New York) and then sterilized by exposure to gamma radiation (2.5 Mrads). Grant et al., 1973, has shown that gamma radiation does not significantly alter crosslinked collagen. In his experiments, the collagen retained its native reactivity and banding pattern.

2.4 CROSSLINKING PROCEDURES

2.4.1 CROSSLINKING PROCEDURE 1

Collagen sheets prepared from Sections 2.1 and 2.2 were immersed in a solution of N-hydroxysuccinimide (N-H) (Sigma Chemical Co., St. Louis, Mo.) and cyanamide (CA), a carbodiimide, (Sigma Chemical Co., St. Louis, Mo.) in a 2:1 weight ratio each to collagen in 40 mls phosphate buffered saline (PBS) (phosphate buffered saline made of 0.3 M NaCl, 18.2 mM Na_2HPO_4 and 2.3 mM NaH_2PO_4 at pH 7.4) per collagen sheet (Figure 2.2). The collagen sheets immersed in this solution were allowed to stand 24 hours at room temperature. The sheets were then washed in distilled water several times and allowed to air dry. This was the control group for comparisons of how acylation of the collagen improved crosslinking.

FIGURE 2.2 Chemical crosslinking reaction in Procedure 1. The method of carbodiimide crosslinking from Weadock, 1983.



2.4.2 CROSSLINKING PROCEDURE 2

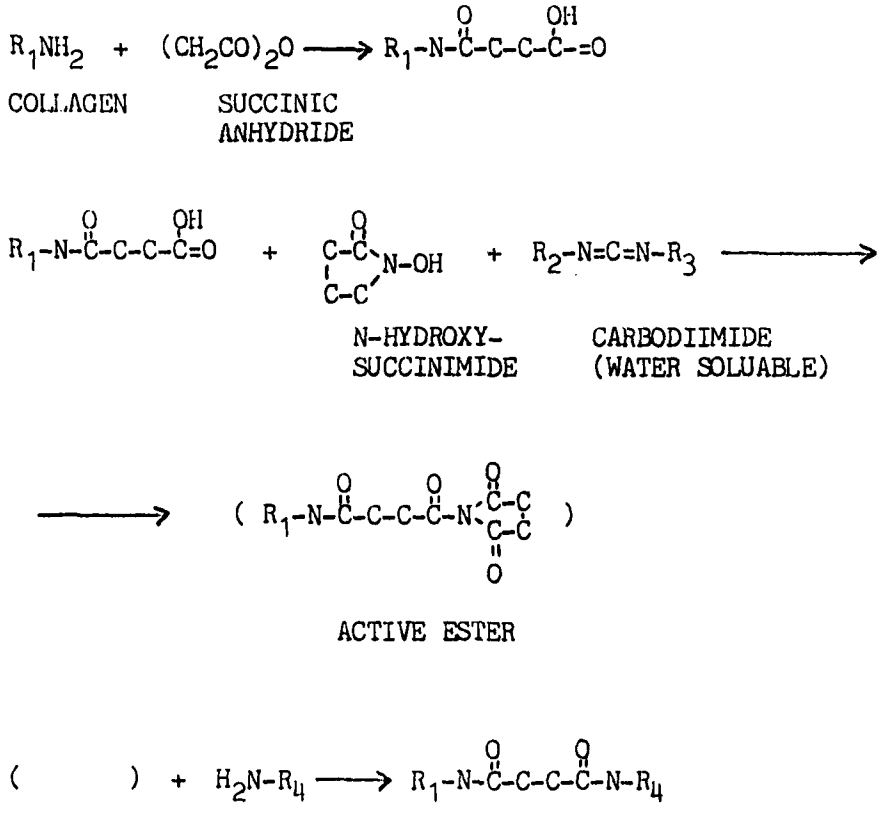
The free amino groups of collagen were acylated using succinic anhydride (SA) (Sigma Chemical Co., St Louis, Mo.). SA was used in a 61 equivalents/free amino group in collagen. Collagen contains approximately 420 free amino groups per collagen molecule; therefore, 9.0 gms of SA were added per gm of collagen.

9.0 gms of SA are added to 80 mls of distilled water and stirred on a magnetic stir plate (Mag-mix, Precision Scientific Co., Chicago, Ill.) for 30 minutes at 37° C. Once the SA is dissolved, 10 N NaOH is added until the pH is 7.2; distilled water is then added to make 100 mls of solution. 1.0 gm of collagen is added to this solution and placed in the blender for 2 minutes at high speed. This mixture is allowed to stand at room temperature for 1 hour. After the hour is up, this mixture is placed in a Buchner funnel with #4 Filter paper (Whatman Filter Paper, Whatman Ltd, England) and washed with 100 mls of distilled water while under vacuum. Next the acylated collagen is freeze-dried for future use. It can be stored in this form since it is a stable species.

Once freeze-dried, the collagen is weighed and made into sheets following Sections 2.1 and 2.2. After the sheet has dried, it is crosslinked using a 2:1 N-hydroxysuccinimide and cyanamide each to collagen weight ratio in 40 mls of PBS per sheet (Figure 2.3). The sheet is allowed to stand in this solution for 24 hours. The sheet is then washed in distilled water several times and allowed to air dry. Sheets were made of 0, 25, 50, 75, and 100% acylated collagen, where the other collagen used to make 1% dispersions was the native,

untreated Devro collagen.

Figure 2.3 Chemical crosslinking procedures 2 and 3 presented graphically.



2.4.3 CROSSLINKING PROCEDURE 3

Collagen from Devro is acylated as described in Section 2.4.2 except, that instead of freeze-drying, the washed collagen is removed from the Buchner funnel and placed in the following solution. N-hydroxysuccinimide (N-H) and cyanamide (CA) in a 2:1 weight ratio each to the acylated collagen is placed in 20 mls of PBS per gm of

collagen (assume all of the collagen is recovered from the wash step). To this solution is added 5 N NaOH until the pH is 7.2. Then the collagen is stirred in using a glass stirring rod and allowed to stand for 3 hours at room temperature (Figure 2.3)

After 3 hours the mixture is washed in a Buchner funnel with #4 Whatman filter paper with 100 mls of distilled water. This treated collagen must be made into sheets or sponges immediately and can not be freeze-dried to be used later, as in the case of the acylated collagen. To make sheets, again the same as Section 2.1, a dispersion of collagen is made, and, as in Section 2.2, the sheets are poured. Again in these experiments, 0, 25, 50, 75, and 100% treated collagen sheets were made and tested.

In the case of sponges (Section 2.3), once the dispersion is in the tray, the tray was placed in a 100% humidity chamber for 24 hours to allow the crosslinks to form prior to freezing.

2.4.4 DEHYDROTHERMAL CROSSLINKING

Untreated collagen sheets were placed in a vacuum oven (National Appliance Co., Skokie, Ill.) and subjected to a 200 micron vacuum for 2 hours. The oven temperature was raised to 110° C for 72 hours and the vacuum was decreased to 10 microns. The oven temperature was allowed to decrease to 35° C before the sheets were removed. This procedure will be described by the nomenclature DHT3 (dehydrothermal crosslinking 3 days).

2.4.5 CROSSLINKING BY A COMBINATION OF DHT3 AND PROCEDURE 3

Collagen sheets were crosslinked using a combination of dehydrothermal crosslinking (Section 2.4.4) and crosslinking procedure 3 (Sections 2.4.1, 2.4.2, and 2.4.3) treatments. These procedures will be described by the nomenclature DHT3 (dehydrothermal 3 days) and P3 (Procedure 3).

2.5 COLLAGENASE ASSAY

A 1000 unit/ml collagenase (Type IV Bacterial Collagenase from Clostridium histolyicum, Sigma Chemical Co., St. Louis, Mo.) solution was prepared by adding 1000 units of collagenase (1 unit will liberate peptides from collagen equivalent to 1.0 micromole of L-leucine in 5 hours at pH 7.4 and at 37° C in the presence of calcium ions) to 1.0 ml of a stock buffer solution (10mM Tris and 25 mM $CaCl_2$ in distilled water at pH 7.4).

Samples of collagen to be assayed were weighed to the nearest 0.01 mg, and 250 units of the above solution were added per mg of collagen in 2.0 mls of the stock buffer solution. A micropipette (Finn pipette, Markson Science Inc, Del Mar, Ca.) was used to dispense the required microliters (250 uL/mg collagen) of the collagenase solution into the test tube containing the collagen. The test tube was then placed in a room maintained at 37° C. When the collagen was visibly degraded, the time was recorded and referred to as collagenase resistance time.

2.6 VOLUME FRACTION OF POLYMER DETERMINATION

Samples of collagen were boiled for 2 minutes and then placed between 2 pieces of filter paper. A 1 kilogram weight was placed on top of the filter paper covering the collagen for 20 seconds. The collagen was then weighed, and this weight recorded as the "wet weight."

The collagen was then placed on aluminum foil and put into an oven (Precision Thelco, Model 18, Precision Scientific co., Philadelphia, Pa.) maintained at 110° C for 3 hours. The collagen was then weighed again, and this weight recorded as the "dry weight." The volume fraction of polymer (V_f) was calculated from:

$$V_f = DW/p_c \; / \; ((DW/p_c) + (WW - DW/p_{H2O}))$$

Where: DW = dry weight (mg)

WW = wet weight (mg)

p_c = density of collagen (1320 mg / cc)

p_{H2O} = density of water (1000 mg / cc)

2.7 SCANNING ELECTRON MICROSCOPY STUDIES

Square samples ranging from 2mm x 2mm to 4mm x 4mm of freeze-dried sponge were mounted on aluminum studs with silver paint and coated with a conductive ultra-thin layer of gold in a Polaron E 5300 (Polaron Equipment LTD, Watford, England) freeze-drying ion-sputter apparatus. The surface in contact with the pan side was used in this study since the air side has been shown to be collapsed

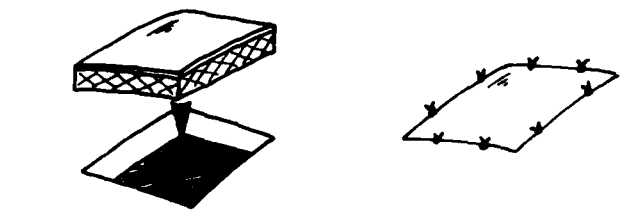
with few pores (unpublished data, this laboratory). These samples were then observed in an Amray model 1400 Scanning Electron microscope (Amray, Bedford, Mass.) using a Lab 6 filament at 30KV. Three areas on each sample were photographed (two sponges were frozen under each condition). Pore sizes were measured and calculated using the calibrated scale on the photograph.

2.8 IN VIVO STUDIES

Collagen sponges made according to Section 2.3 were placed on the backs of Hartley Albino guinea pigs weighing between 300 to 400 gms. The guinea pigs were anesthetized using diethyl-ether (Mallinckrodt, Inc, Paris, Kentucky), shaved, depilatated using Nair (Carter Products, New York, N.Y.) and washed. Prior to surgery, the depilatated skin was washed first with 70% ethanol and then with Betadine solution (Purdue Frederick Co., Norwalk, Conn.). Using aseptic technique, a 2 x 2 cm full thickness excision was removed exposing the panniculus carnosus. After hemostasis, a collagen sponge, cut the same dimensions as the wound, (rehydrated in sterile PBS solution) was implanted. The silicone layer was sutured in place by 8 interrupted threads of 4/0 chromic gut suture material (Ethicon, Inc, Sommerville, New Jersey; see Figure 2.4). The animals were wrapped in sterile gauze and then in an elastic bandage, Elastikon (Johnson and Johnson Products, New Brunswick, N.J.). The animals were fed with a normal diet and water ad libitum. The animals were sequentially sacrificed at 6, 9 and 12 days post implantation. Under anesthesia, a sample containing the wound and the edges of normal skin

was excised. The specimen was fixed using Carson's fixative, dehydrated through a graded series of alcohols, cleared in xylene, infiltrated and embedded in paraffin. The paraffin blocks were sectioned at 5um and stained with Hemotoxylin and Eosine (H&E) or with Masson's Trichrome and observed using transmitted light microscopy.

Figure 2.4 Diagram depicting the 2 x 2 cm full thickness excision and suturing process that is used for in vivo studies on guinea pigs.



3. RESULTS

3.1 CROSSLINKING STUDIES

1% BHC sheets, made in sections 2.2 and 2.4 were characterized using two tests. Collagenase resistance assays and volume fraction of polymer analysis were used to compare the extent of crosslinking of the various sheets. Tables 3.1 through 3.5 present results obtained from these investigations. The n values are shown for each table. So the levels of significance could be analyzed, a one-end tailed t-test was applied, since only the lower limit was of concern.

For the purposes of convenience, the following abbreviations were used:

BHC	-BOVINE HIDE COLLAGEN
CRT	-COLLAGENASE RESISTANCE TIME
V_f	-VOLUME FRACTION OF POLYMER TEST
M	-SAMPLE MEAN
STD	-SAMPLE STANDARD DEVIATION (n-1) (+/-)
P(n)	-CROSSLINK PROCEDURE 1, 2 OR 3
DHT3	-DEHYDROTHERMAL CROSSLINKING FOR 72 HOURS
C1	-EXPOSURE TO 1% CARBODIIMIDE FOR 24 HOURS

3.1.1 CONTROL

Table 3.1 illustrates the properties of uncrosslinked and C1DHT3 1% BHC sheets, which were used to compare with the results of the new

crosslinking technique.

3.1.2 CROSSLINKING PROCEDURE 1

Table 3.2 illustrates the properties of uncrosslinked 1% BHC sheets exposed to a solution of N-hydroxysuccinimide (N-H) and cyanamide (CA) in a 2:1 weight ratio of N-H and CA each to collagen. The V_f properties of P1 were significantly greater than uncrosslinked collagen, though the CRT was the same. However, P1 properties were less than C1DHT3 crosslinked sheets.

3.1.3 CROSSLINKING PROCEDURE 2

Table 3.3 illustrates the properties of acylated collagen made into 1% sheets. The acylated collagen was mixed with untreated collagen in varying percentage amounts prior to dispersion. After the sheets were dry, they were crosslinked using a 2:1 weight ratio of N-H and CA to collagen sheet. The V_f properties after crosslinking of the acylated collagen sheets were significantly greater than the uncrosslinked collagen sheets but they were still less crosslinked than the C1DHT3 crosslinked sheets.

3.1.4 CROSSLINKING PROCEDURE 3

Table 3.4 illustrates the properties of acylated collagen treated with N-H and CA prior to being made into sheets. Only the P3 100% sheets were significantly greater than the uncrosslinked collagen and were equivalent to C1DHT3. Based on these results, material crosslinked with a combination of procedure 3 and DHT3 was prepared.

Table 3.1 Properties of control sheets of 1% uncrosslinked BHC and C1DHT3 sheets. Each value is the average of 9 samples (n=9).

	CRT (hrs)		V_f	
	M	STD(±)	M	STD(±)
1% BHC Unc	2.67	0.76	0.0552	0.0087
1% C1DHT3	24	1.00	0.381	0.030

Table 3.2 Properties of 1% BHC sheets before and after crosslinking Procedure 1. Each value is the average of 9 samples (n=9).

	Before				After			
	CRT (hrs)		V_f		CRT (hrs)		V_f	
	M	STD (±)	M	STD (±)	M	STD (±)	M	STD (±)
1% BHC	2.67	0.76	0.0552	0.0087	2.67	1.0	0.229	0.018

Table 3.3 Properties of 1% acylated collagen sheets before and after crosslinking Procedure 2. Each value is the average of 9 samples (n=9).

	Before				After			
%acylated collagen	CRT (hrs)		V_f		CRT (hrs)		V_f	
	M	STD (±)	M	STD (±)	M	STD (±)	M	STD (±)
0	1.5	0.43	0.0674	0.0038	1.5	0.25	0.203	0.020
25	2.08	0.29	0.0683	0.0039	5.42	0.29	0.186	0.026
50	1.92	0.38	0.0900	0.0077	13.0	0.25	0.205	0.0098
75	2.33	0.29	0.0603	0.012	2.33	0.38	0.193	0.0095
100	2.83	0.29	0.0415	0.0034	3.00	0.43	0.212	0.021

Table 3.4 Properties of collagen sheets made using crosslinking Procedure 3. Note: There is no before and after crosslinking because the crosslinking takes place before the sheet is formed. Each value is the average of 9 samples (n=9).

%acylated collagen	CRT (hrs) M	STD (±)	V_f M	STD (±)
0	2.67	0.76	0.0552	0.0087
25	1.5	0.25	0.0501	0.0060
50	1.5	0.25	0.0475	0.0080
75	1.67	0.14	0.0426	0.0020
100	14.0	0.25	0.396	0.060

Table 3.5 Properties of collagen sheets made using crosslinking Procedure 3 (P3) with 100% acylated collagen (100 %) then DHT3 (P3 100% DHT3). Each value is the average of 9 samples (n=9).

%acylated collagen	CRT (hrs) M	STD (±)	V_f M	STD (±)
100	53.0	12.8	0.580	0.074

3.1.5 COMBINATION OF DEHYDROTHERMAL AND PROCEDURE 3 100% CROSSLINKING

Table 3.5 illustrates the properties of P3 100% DHT3 crosslinked sheets. These sheet's properties inferred they were significantly more crosslinked than C1DHT3 sheets.

3.2 SCANNING ELECTRON MICROSCOPY STUDIES

Two variables were used to study the pore size/meshwork of collagen sponges. In the past, pore size has been controlled by varying the concentration (w/v) of collagen-acid dispersions (M.S. Thesis by E. Chen, 1982). Dagalakis et al. (1980) changed the pore structure by variation of the freeze-drying conditions. In this study, the pH of the dispersion and the freezing bath temperature were varied. Table 3.6 illustrates one of the freezing experiments conducted, where the freezing bath temperatures were varied. The samples were all one pH and dispersion, P3 100%, 0.5% collagen sponge. Table 3.7 illustrates one of the freezing experiments conducted, where temperature and pH of P3 100%, 0.5% collagen sponges were varied. The sponges were primarily fibrous in nature and very open. Table 3.8 illustrates a summary of pore size at the extreme ends of the freezing experiments of the type in Table 3.6. Figure 3.1 depicts the difference of the sponges under these extreme conditions. In Figure 3.1 A, the meshwork is finer and closer structured as the collagen dispersion is frozen faster (cooler bath temperature) than Figure 3.1 B. Figure 3.2 shows the difference between the -40°C bath and -30°C metal plate. The ethanol bath, because there is no air gap between the boat and the cold surface, freezes the dispersion much faster.

Table 3.6 Freezing Experiment (FE) I, pH 3.5 dispersion of P3 100%, 0.5% collagen sponges frozen at various temperatures. The temperatures listed under the column of temperature of bath were the temperatures at which each of the two sponges were frozen. The first temperature column is what temperature those two sponges were grouped under. The comments column indicates whether the sponge cracked or was nonuniform.

TEMP (° C)	TEMP OF BATH (° C)	# OF SPONGES FROZEN	COMMENTS
-90	-92, -92	2	BOTH CRACKED
-80	-83, -80	2	1 CRACKED
-70	-71, -73	2	1 CRACKED
-60	-61, -63	2	BOTH UNIFORM
-50	-52, -51	2	BOTH UNIFORM
-40	-44, -44	2	BOTH UNIFORM
-30	(no bath used)*	2	BOTH NONUNIFORM

* -30 was frozen on the freezer shelf without an ethanol bath.

Table 3.7 Freezing Experiment (FE) II, pH and temperature of the ethanol bath was varied while freezing a P3 100%, 0.5% collagen dispersion. The temperature of the bath is the temperature when each sponge was placed in the ethanol bath. The temperatures listed are for each sponge's starting temperature. The first temperature column is the temperature at which the sponges are grouped. The comments column indicates whether the sponges cracked or was not uniform.

pH	TEMP (° C)	TEMP OF BATH (° C)	COMMENTS	# OF SPONGES FROZEN
2.13	-80	-81, -83	1 CRACKED	2
2.13	-55	-57, -58	BOTH UNIFORM	2
2.13	-30	-30, -35	BOTH NONUNIFORM	2
2.5	-80	-81, -82	BOTH UNIFORM	2
2.5	-55	-57, -58	BOTH UNIFORM	2
2.5	-30	-30, -35	BOTH NONUNIFORM	2
2.72	-80	-81, -83	BOTH CRACKED	2
2.72	-55	-57, -57	BOTH UNIFORM	2
2.72	-30	-31, -35	BOTH NONUNIFORM	2
3.0	-80	-82, -83	1 CRACKED	2
3.0	-55	-57, -60	BOTH UNIFORM	2
3.0	-30	-31, -33	BOTH NONUNIFORM	2
3.28	-80	-80, -83	1 CRACKED	2
3.28	-55	-58, -60	BOTH UNIFORM	2
3.28	-30	-35, -33	BOTH NONUNIFORM	2

Table 3.8 Summary of freezing experiment I, pH 3.5, 0.5% collagen sponges. Pore sizes were measured and calculated from the calibration bar on the three photographs of each specimen. These numbers are an estimate of the average size of the predominant pore structure found in each sample at a particular temperature. Each size is the average of 6 samples (n=6).

TEMP (° C)	SIZE (uM)	CONDITIONS UNDER WHICH FROZEN
-90	14	ETHANOL BATH
-40	28	ETHANOL BATH
-30	110	FREEZER SHELF W/O BATH

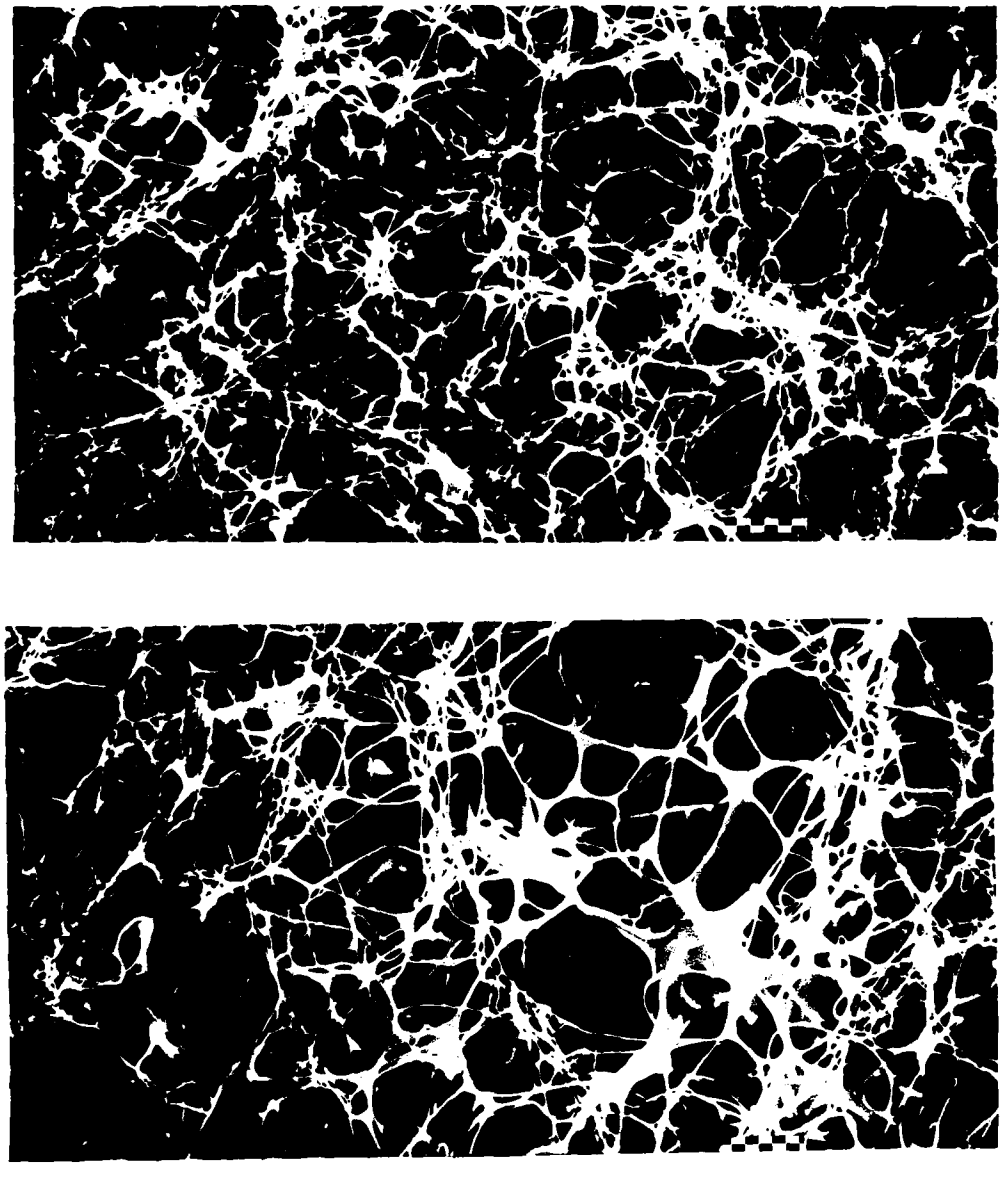


Figure 7.1 Two 0.5% sponges of P3 100% crosslinked collagen. Sample A (top) was frozen at -90° C, while sample B (bottom) was frozen at -40° C. The magnification is X1560, and the calibration bar for both pictures is 7 um long.

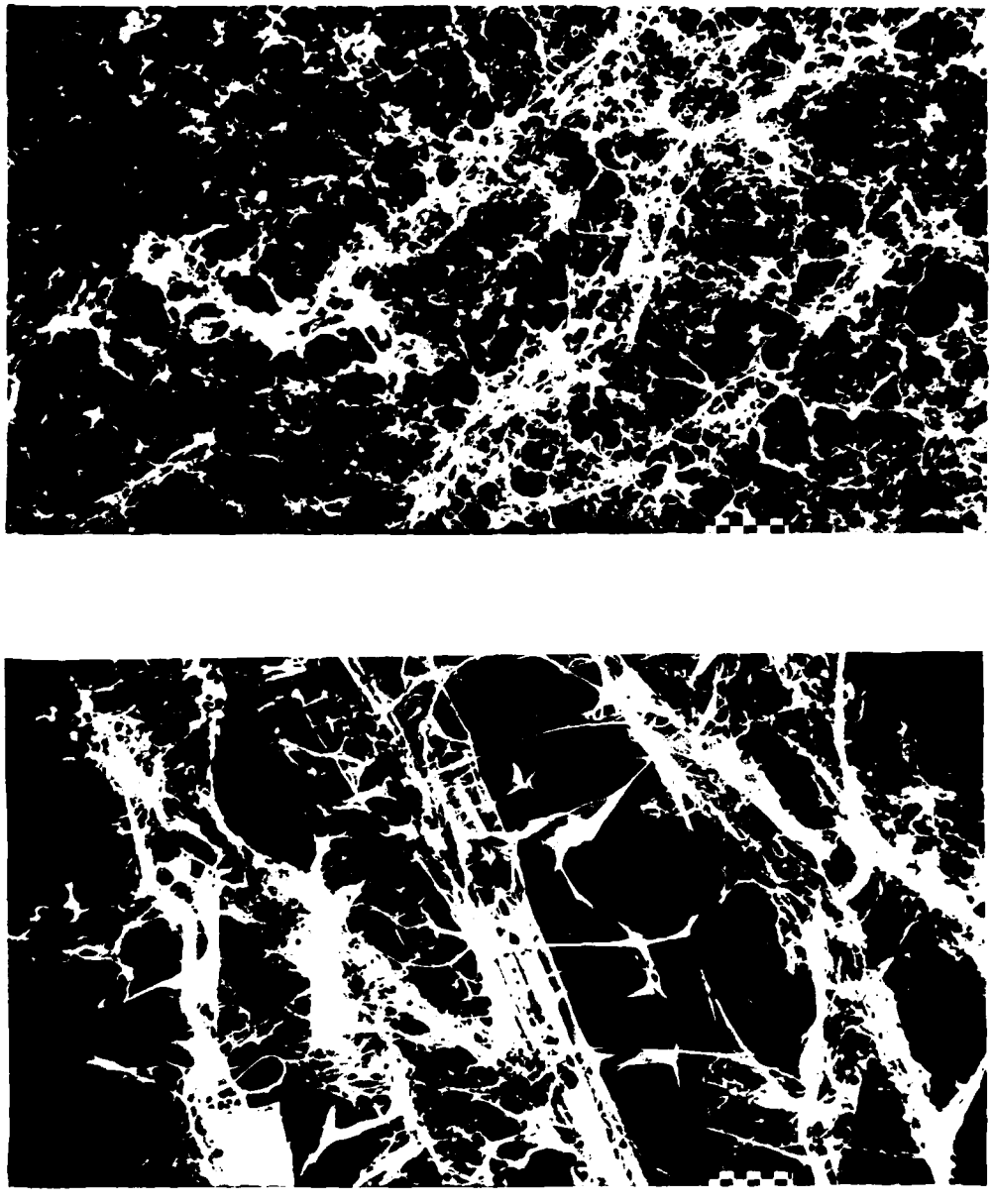


Figure 3.2 Two 0.5% sponges of P3 100% crosslinked collagen. Sample A (top) was frozen at -40° C in an ethanol bath, while sample B (bottom) was frozen at -30° C on a metal plate. The magnification for both pictures is X312, and the calibration marker is 36 um long in both pictures.

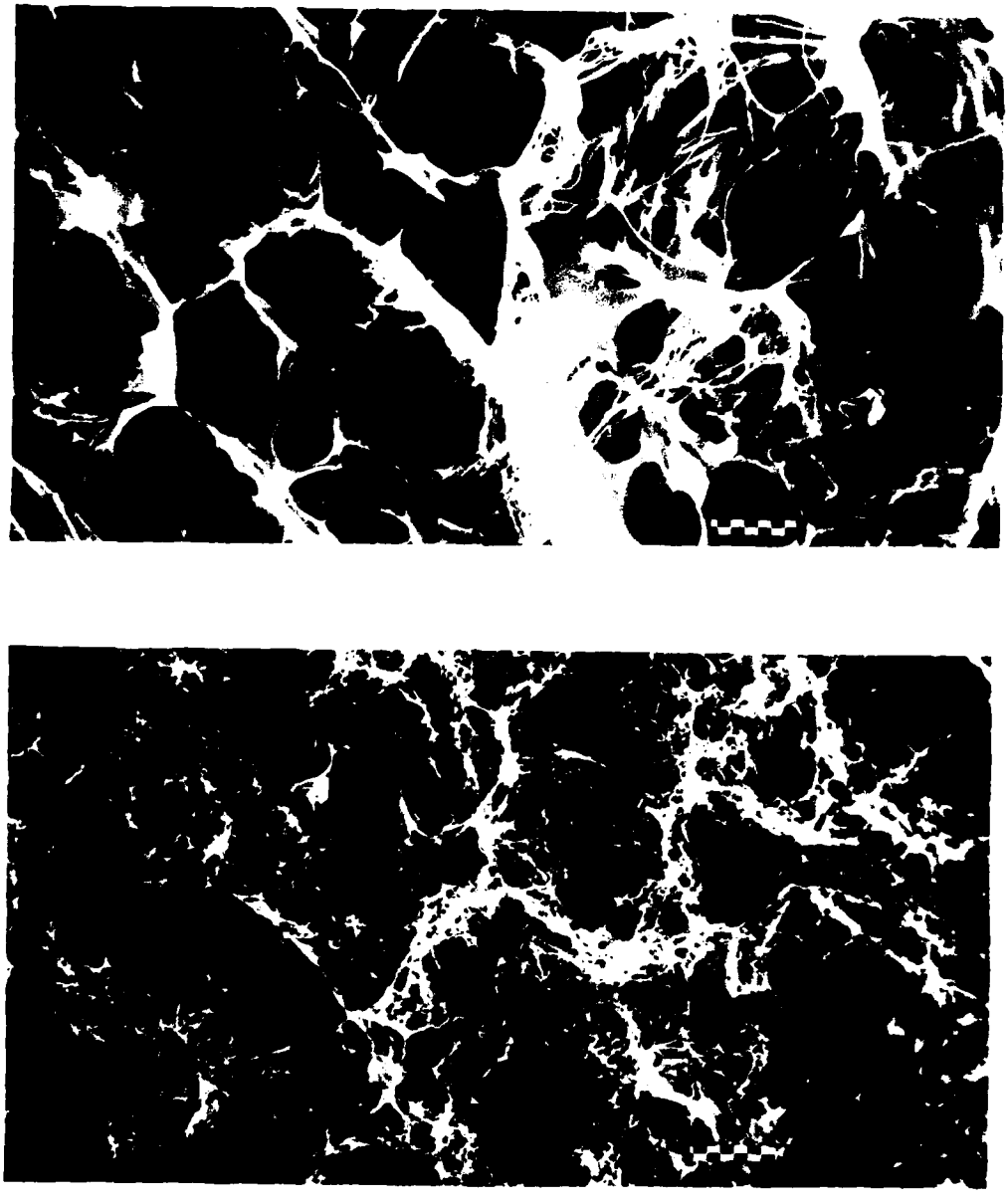


Figure 3.3 Sample A (top) is pH 2.0 1% uncrosslinked collagen sponge frozen at -30° C in an ethanol bath. Sample B (bottom) is pH 3.25 1% uncrosslinked collagen sponge frozen under the same conditions. Magnification for both is X312, and the calibration marker is 36 um for sample A and 39 um for sample B.

Figure 3.3 depicts the difference between the different pHs at the same temperature. A pH of 2.0 tends to form more porelike structures, but the higher pH (3.5) is more fibrous and has a less organized structure overall. Table 3.9 illustrates the freezing of sponges of different pHs, at three bath temperatures. At the warmer temperatures, as the pH increases, so does the average meshwork openings. Table 3.10 shows the mean size of the meshwork of these same sponges averaged at each temperature. For these calculations the pH of the sponges was not considered.

Uncrosslinked 1% sponges showed the same basic features as the P3 100%, 0.5% collagen sponges. One difference noted was the pore sizes and structures were smaller which occurs in higher concentrations as previously shown by Chen (1982). Figure 3.4 shows pH 2.0 and pH 3.75 dispersion sponges frozen under the same conditions; they were frozen on a metal plate at -30° C. Figure 3.5 shows dispersion sponges prepared at pHs of 2, 3 and 3.75 and frozen at -55° C in an ethanol bath. The differences in sponge structures are still present, but less pronounced. The different meshwork openings that characterize these sponges gradually change as the pH is varied. A larger amount of amorphous material was observed in the sponge prepared at pH 2.0. This phenomenom, which causes the sponge pores to be smaller is not understood.

Table 3.9 Freezing experiment II, Mean and Standard Deviation of meshwork openings as a function of pH and freezing temperature. Each number is the average of 15 samples (n=15).

pH	TEMP	MEAN PORE SIZE (um)	STD DEV (±)	COMBINED AVERAGE FOR A pH MEAN PORE SIZE (um)	STD DEV (±)
2.13	-80	14.25	1.06		
2.13	-55	25.25	3.89	28.0	14.2
2.13	-30	44.5	7.78		
2.5	-80	15.38	0.530		
2.5	-55	22.5	0.500	28.4	15.8
2.5	-30	47.25	11.7		
2.72	-80	16.25	1.77		
2.72	-55	27.25	6.72	30.8	16.3
2.72	-30	48.75	13.8		
3.0	-80	15.5	0.707		
3.0	-55	29.1	4.07	37.6	25.3
3.0	-30	68.3	13.8		
3.28	-80	15.5	0.707		
3.28	-55	33.0	4.24	38.9	24.9
3.28	-30	68.3	13.8		

Table 3.10 Freezing experiment II, Mean and Standard Deviation of meshwork openings when only considering the temperature a sponge was frozen at and not its pH. Each mean size is the average of 75 samples (n=75).

TEMP °C	MEAN SIZE um	STD DEV (±)
-80	15.4	1.04
-55	27.4	4.95
-30	55.4	14.5

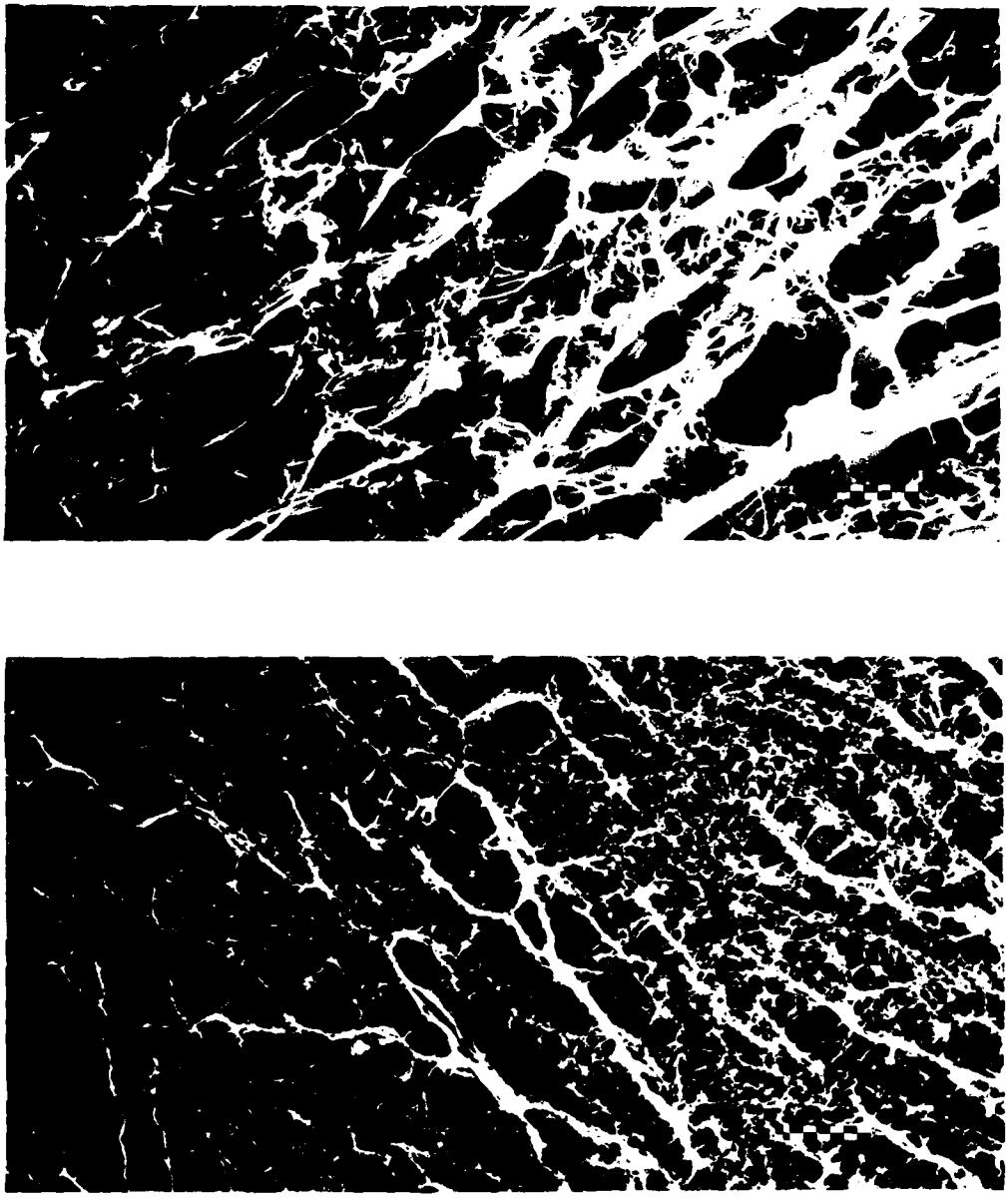


Figure 3.4 Sample A (top) is a pH 2.0 1% unerosslinked sponge frozen at -30° C on a metal plate. Sample B (bottom) is pH 3.[illegible] 1% unerosslinked sponge frozen under the same conditions. Magnification is X312, and the calibration bar marker is 39um for both samples.

Figure 3.5 Sample A (top) is a pH 2.0, 1% uncrosslinked collagen sponge frozen at -55° C in an ethanol bath. Sample B (middle) is a pH 3.0, 1% uncrosslinked collagen sponge frozen under the same conditions. Sample C (bottom) is a pH 3.75 1% collagen sponge frozen under the same conditions. Magnification is X780 for all three samples. The calibration bar markers for A and B are each 15um long and the calibration bar for sample C is 16um long.

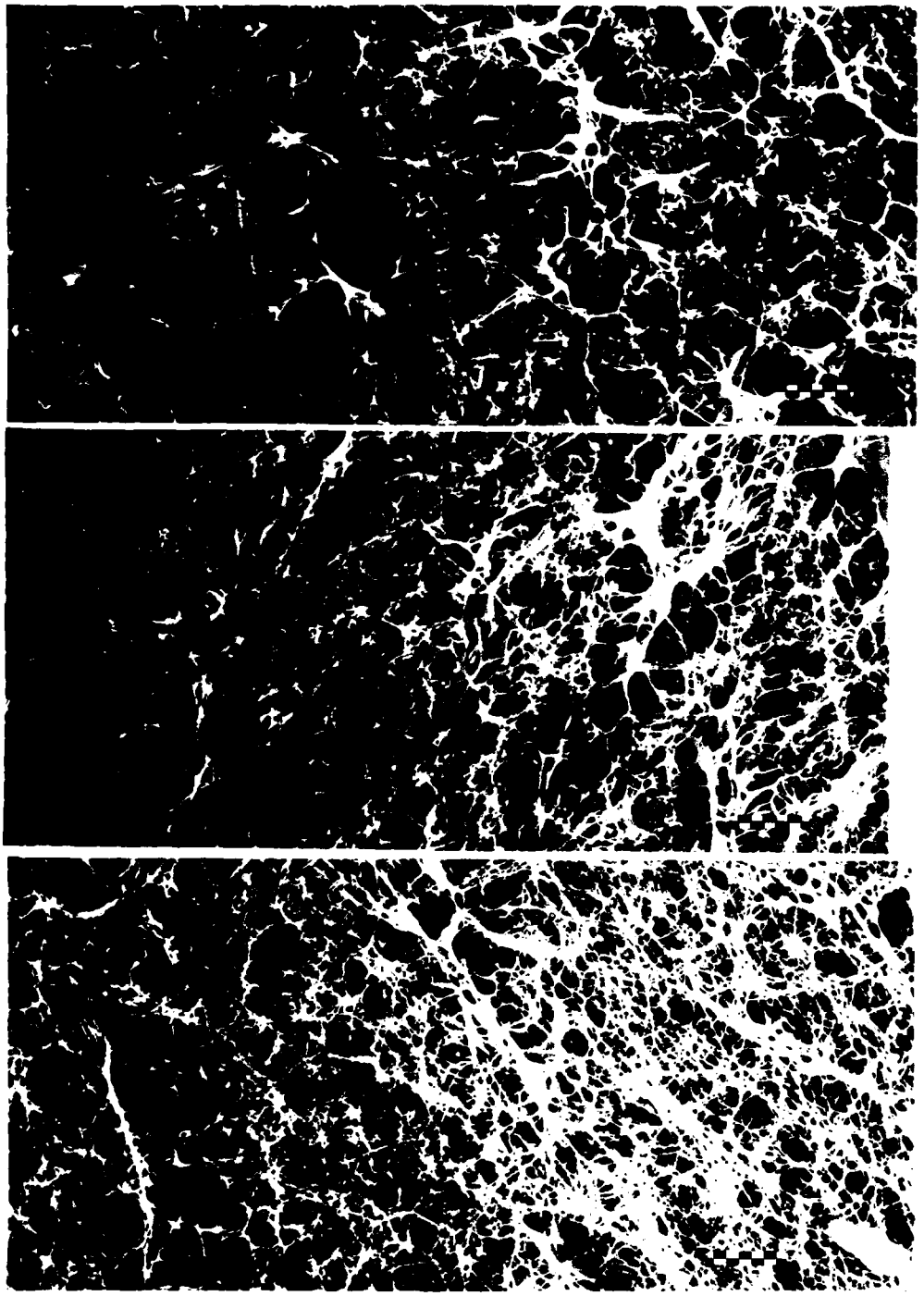


Figure 3.5

3.3 IN VIVO STUDIES

In vivo studies using P3 100% and P3 100%DHT3 crosslinked sponges are currently being conducted in collaboration with Dr. Charles Doillon. The initial results from the sponges provided to Dr. Doillon indicate the sponge is accepted by the host tissue with many fibroblasts migrating into the sponge. Figure 3.6 depicts the fibroblast migration into the sponge by day 12. Dr. Doillon's results also indicate that the results obtained with P3 100%DHT3 sponge are not statistically different from the C1DHT3 sponges. When the two procedures are compared using the number of fibroblasts and leukocytes present in the sponge after 6, 9, and 12 days post implantation and the amount of brightness measured in polarized light microscopy (which indicates newly deposited collagen), there was no statistical difference. This infers the two procedures are interchangable.

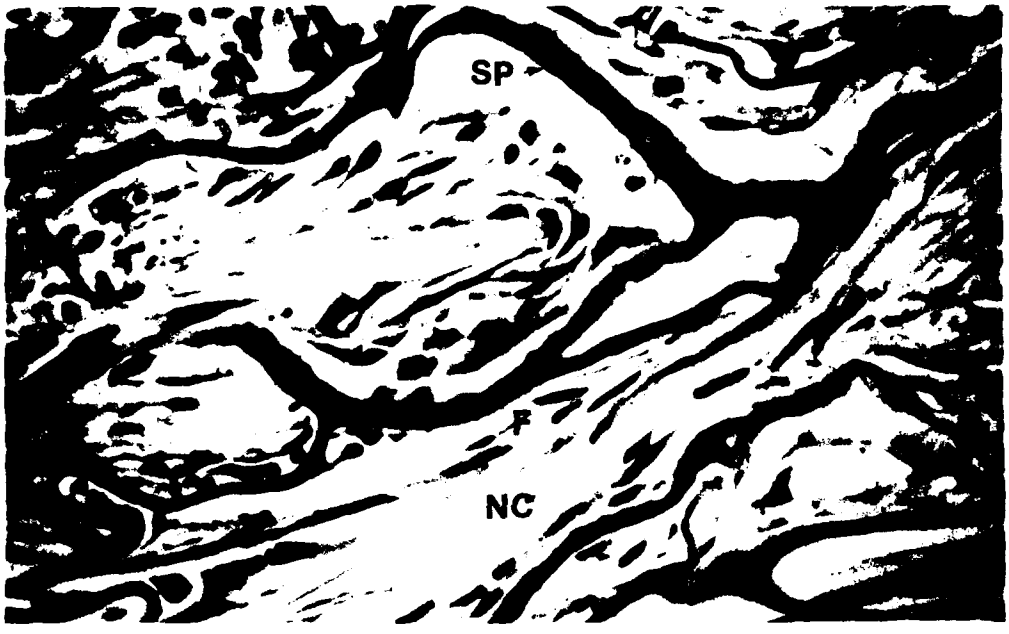


Figure 3.6 This is a histological slide of P3 100%, 0.5% sponge at day 12 post implantation. This sponge has 1% hyaluronic acid and 1% fibronectin added as chemoattractants. The sponge (SP), the dark strands, is well invaded with migrating fibroblasts (F). There is also a large amount of new collagen (NC) being laid down. This slide is stained with hemotoxylin and eosine (H&E). The magnification is X1845. Picture taken by Dr. Charles Doillon.

4. DISCUSSION

4.1 CROSSLINKING STUDIES

Crosslinking of collagen sponges to retard their resorption rate in vivo is a crucial design feature of an artificial skin. Previous work by White et al. (1973) has shown that collagen films become more resistant to both bacterial and mammalian collagenases as crosslinking was increased. Chvapil (1977) extended this research when he showed the in vivo rate of resorption of extruded collagen tubes could be controlled through crosslinking. A definite inverse relationship between crosslink density and resorption rate was established. Chen (1982) has shown, based on histological sections, that the uncrosslinked full thickness grafts were resorbed much more quickly than their crosslinked counterparts. Therefore crosslinking has to be considered a most important step when fabricationg a sponge for in vivo implantation. But crosslinking does not only retard resorption rate, it is also a useful method of binding sponge additives such as fibronectin and hyaluronic acid (HA), two cell chemoattractants, to the collagen scaffold. Crosslinking of fibronectin and HA to collagen prevents rapid elution of these materials into the physiological fluid of the wound. A much slower controlled release can be accomplished. Crosslinking is also a method of adding strength to the sponge for use in the surgical environment, such as suturing the sponge to the wound surface (Yannas, Burke, Gordon et al., 1980). Based on the above

reasoning, crosslinking will remain a crucial point in the design of an artificial skin.

Other researchers have used glutaraldehyde as the main crosslinking agent of their collagen biomaterials (Yannas, Burke, Gordon et al., 1980). While glutaraldehyde has been proven as an effective crosslinking agent, several problems must be discussed. If crosslinking solutions of glutaraldehyde are too concentrated, the glutaraldehyde can coat collagen fibers in the form of long polymers and, therefore, be capable of depleting free glutaraldehyde from the solution. This action would make the glutaraldehyde unavailable for further penetration into the sponge, creating an uneven distribution of crosslinks in the sponge (Cheung and Nimni, 1982). Such incomplete or heterogenous crosslinking would prove to be a significant problem, allowing the implant to be more susceptible to degradation by collagenase once the inner matrix was exposed.

Another problem of glutaraldehyde treated collagen sponge is the spontaneous release of glutaraldehyde in vivo. Speer et al. (1980) showed that the spontaneous release of free glutaraldehyde from the sponge had cytotoxic effects upon fibroblasts in cell tissue culture. Foreign body giant cell immunoreactions to the sponges occurred in vivo. Cooke et al. (1983) showed glutaraldehyde treated sponge to be cytotoxic to cells in tissue culture. These results correlated with earlier work done by Oliver et al. (1976). His work showed that when collagen was treated with glutaraldehyde in higher concentrations, it suppressed collagenase activity in his in vitro assay and gave increasingly pronounced and persistant inflammatory response in vivo.

Along with this inflammatory response were increased foreign body reactions and the persistant presence of multinuclear giant cells.

Contrary to the above findings, Burke et al. (1981) has reported success with glutaraldehyde crosslinked collagen based dermal equivalents. He reported finding no host-graft rejection along with no histologic evidence of an immunologic reaction. The differences in these findings must be the method of preparation. Burke's group used only 0.05% w/v glutaraldehyde solution with repeated washings to remove any residual glutaraldehyde. This is well below the 4% w/v solution that Oliver et al. (1976) used. But if one chooses not to use glutaraldehyde because of possible side effects, several other effective alternatives exist.

Chvapil (1982) has studied hexamethylene diisocyanate, a powerful crosslinking agent that is toxicologically acceptable. He changed to this new crosslinking agent after his experiments showed that the glutaraldehyde treated sponge had more cellular reaction and less cell infiltration than the hexamethylene diisocyanate crosslinked sponge (Chvapil et al., 1983). Unlike glutaraldehyde, hexamethylene diisocyanate does not leave any residue within the sponge. The product of the crosslinking reaction is extractable, and the agent does not polymerize (Chvapil, 1982).

Weadock (1983) characterized another crosslinking method. A combination of dehydrothermal (DHT) crosslinking and cyanamide (carbodiimide) proved to act synergistically to crosslink a collagen sponge. His in vivo studies have also shown that the crosslinked sponge is biologically acceptable. This thesis explored yet another

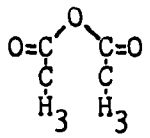
method of crosslinking.

4.2 PROCEDURES

Olcott (1947) listed acetic anhydride as a group specific reagent for amino groups on proteins. Green et al. (1953) reported that acetic anhydride was a specific means for the N-acylation of proteins, more specifically, collagen. Kishida et al. (1975) took the acylation of protein one step further in that succinic anhydride was used to acylate a marker for crosslinking to an antibody for use in electron microscopy. This was accomplished through the use of a water soluable carbodiimide and N-hydroxysuccinimide (N-H) to form an active ester of the acylated group. The structures of acetic anhydride and succinic anhydride are in Figure 4.1.

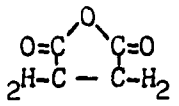
Figure 4.1 Comparison of acetic and succinic anhydrides.

ACETIC ANHYDRIDE



$(CH_3CO)_2O$

SUCCINIC ANHYDRIDE



$(CH_2CO)_2O$

Notice the similarity of the two compounds. Since this acylation-esterization process formed covalent bonds between markers and antibodies, the thought occurred to try to develope a crosslinking method to be used to covalently crosslink collagen to itself. Figure

2.3 shows the reaction that occurs in this crosslinking method. Originally the idea was to mix untreated collagen with the active ester collagen to form the crosslinks. However under the experimental conditions used, the most crosslinks were formed using pure treated collagen in procedure 3 (P3). P3 materials were then characterized and compared to Weadock's (1983) C1DHT3 method.

4.3 COMPARISON OF METHODS

Procedure 3 crosslinked materials were statistically similar to Weadock's (1983) C1DHT3 materials; however, C1DHT3 materials resisted collagenase attack better. Once P3 materials were crosslinked also with DHT3, they were significantly more crosslinked than the C1DHT3 as determined by collagenase resistance assay and volume fraction of polymer test (see Tables 3.1 and 3.5). These findings add credence to the evidence that this method enhances the crosslinking of collagen. Presently, in vivo studies underway suggest that while P3 and C1DHT3 are not significantly different in vivo, preliminary results indicate that incorporation of HA and fibronectin additives using the P3 method may give statistically better results; more full thickness excision implantations must be done by this lab before the data is statistically significant.

4.4 SCANNING ELECTRON MICROSCOPE STUDIES

As important as crosslinking is to the biodegradation rate in the implant in vivo, sponge surface structure may be just as critical in fabricating an optimum artificial skin.

When Ehrman and Gey (1956) showed enhanced cell growth in cell cultures using collagen gels, an in vitro cell culture revolution was born. Since then, numerous studies have shown how well cells grow on collagen gel substratum compared to other cell culture surfaces such as plastic, glass and even collagen films. Gey et al. (1974) reported the clear difference collagen substrate made when growing chick cells. The cells grew better and longer on collagen than on glass. Bard and Hay (1975) have cultured corneal fibroblasts on collagen gels. They found that the fibroblasts are morphologically and morphogenetically more similar to cells in vivo than they are to cells cultured on glass or plastic.

The extracellular matrix is of great importance in the formation and maintanence of correct tissue morphology and the regulation of morphogenetic events and cell mobility (Ruoslahti and Engvall, 1980). Hydrated collagen gels provide a substratum not unlike the in vivo environment. The gels contain collagen in a fibrillar form. Collagen in a fibrillar form is preferred by cells because of the unique properties of the surface of collagen fibers (Chvapil, 1977). With the high content of both diamine and dicarboxylic amino acids and a carbohydrate moiety, the collagen molecule has a rather rough molecular surface (Chvapil, 1977). The fact that fibronectin, a cell attachment protein, is not required to attach cells to hydrated collagen gels emphasizes the possible importance of the interplay between the physical organization of the substratum and other factors (Grinnel, 1982). Dunn and Eberdal (1978) showed a contact guidance between cells and the collagen gel matrix. By using an oriented

collagen gel matrix, they succeeded in growing fibroblasts in an aligned arrangement. Overton and Collins (1976) showed the mesenchymal network of collagen in the developing chick to be a very close, tightly knit structure of random fibers around the cells. Based on all the above evidence, the need for a fibrous surface for the artificial skin is a logical conclusion. The surfaces of the collagen sponges were studied by changing the conditions in which the collagen sponges were made.

4.4.1 CONDITIONS OF SPONGE PREPARATION

In the past pore size has been controlled by varying the concentration of the collagen-acid dispersion (Chen, 1982). Another method used by Dagalakis et al. (1980) was the changing of freeze-drying conditions. Another approach has been explored in this thesis, that is the variation of pH of the collagen-acid dispersion and the temperature of freezing. By freezing the collagen dispersions at different temperatures while keeping the pH of the dispersion constant, the effect of fast or slow growing water crystals could be better evaluated. After establishing the effects of slow versus fast freezing, pH was varied to see its role in the development of the freezing collagen sponge surface.

4.4.2 FREEZING EFFECTS

Fast freezing of collagen sponges at temperatures less than -80° C invariably cracked the sponge during freezing. The freezing process was too fast and an intact sponge could not be made. But the sponges made at these temperatures were very uniform and tightly knit fibrous structures. The average meshwork opening was approximately 3 to 15 um depending on the concentration of the collagen (3-5um for 1% and 6-15um for 0.5% dispersions). An example of this type of sponge is in Figure 3.1 A.

Fast freezing at temperatures between -80° C and -60° C were also very uniform and tightly knit fibrous structures. However if the sponge was kept in the freezing bath after it was solid, cracking of the sponge occasionally occurred.

Freezing sponges at temperatures between -60° C and -40° C never cracked. These sponges were very uniform and still retained most of the fibrous structure (depending on the pH of the dispersion).

Slow freezing of sponges was accomplished at temperatures between -40° C and -20° C. Freezing in this range proved to be an erratic situation. The sponges proved to be very sensitive to minor changes in the freezing environment. Frost on the freezing plate, freezer shelf surface designs, and small holes in the metal plate, all left their imprint on the bottom side of the sponges. Uniformity was a significant problem in this range. Large ice crystals grew since the water in the hydrated collagen dispersion had time to diffuse through the dispersion to concentrate at the site of the crystal, thereby,changing the structure of the sponge (Chen, 1982). This

allowed certain areas of the collagen to condense into amorphous sheets (no collagen fibers were observed up to X24,000 magnification on the SEM when viewing these structures).

In these experiments, the average size of the meshwork openings doubled from 14um to 28um when the freezing temperature of the ethanol bath was changed from -90° C to -40° C (P3 100%, 0.5% collagen dispersions were used). However, when the freezing temperature was raised another 10° C to -30° C, the meshwork openings approximately doubled again to 50um.

These experiments inferred that when the sponge is frozen faster, it will be more fibrous and have smaller pores.

4.4.3 VARIATION OF PH EFFECTS

Variation of the pH was shown to affect pore structure. A literature search failed to find experiments performed in this area. The pH effect was noticeable at -55° C as shown by Figure 3.5. However, at -30° C, the pH proved to be a significant factor as to what the sponge surface would look like. Figure 3.3 is a typical example. The pH 2.0 sponge has some fibers present, but the sponge consists mainly of amorphous sheetlike structures that form pores. Under the same freezing conditions the pH 3.25 sponge is much more fibrous, less organized in appearence and has much less of the amorphous material present. The cause of the formation of the amorphous material is not understood. It must be a condensation of tightly packed collagen fibers, but the SEM could not show any structural details, even at X24,000 magnification.

As the pH is varied from 2.0 to 3.75, the sponge surface characteristics change from porous sheetlike structures to meshes of collagen fibers respectively. While the mechanism is not understood, the results can be used to control the surface of sponges used in biological dressings.

4.5 FINDINGS

Based on this research, a new crosslinking method has been characterized, which is sufficiently crosslinked to provide a collagen-based dermal equivalent. In addition to this, the sponge surface characteristics and the control of these characteristics has been documented. Whereas Yannas and Burke (Yannas et al., 1980) have a dermal equivalent sponge which does well in vivo (Burke et al., 1981), by using these new procedures that have been characterized here, a better dermal equivalent could possibly be made. This new dermal equivalent is more fibrous and open meshed, and is more like the in vivo extracellular matrix than the Yannas and Burke dermal equivalent. Hopefully the future in vivo experiments will support these conclusions.

CONCLUSIONS

1. Procedures 1 and 2 inadequately crosslinked the collagen sheets when compared to C1DHT3.

2. Procedure 3 DHT3 provides a densely crosslinked material for use in an artificial skin.

3. Procedure 3 and procedure 3 DHT3 crosslinking methods provide new methods of crosslinking collagen sponges for use in biological dressings.

4. The sponge surface and the conditions that vary it were characterized. A procedure was developed for making a collagen sponge with a surface similar to in vivo conditions. This sponge surface should allow better ingrowth of fibroblasts than previous sponges. An in vivo experiment will be conducted in the near future which will test this proposed sponge.

5. The conditions that provide these optimum features in a sponge are: a) pH range of 3.5 to 3.75; b) freezing temperature of -55° C in an ethanol bath; and c) dispersions of concentrations between 0.5% and 1% collagen, either uncrosslinked or P3 treated.

6. To better quantify fiber density and meshwork structures, a protocol is needed, perhaps in conjunction with a computer program, to

calculate a ratio of open area to fiber surface area in the sponges under the various conditions.

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