SOLID STATE CROSSLINKING

PROCESS FOR

COLLAGEN-GLYCOSAMINOGLYCAN MEMBRANES

by

JAMES FORREST KIRK

SUBMITTED TO THE DEPARTMENT OF MATERIALS SCIENCE AND ENGINEERING IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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ABSTRACT

Research was done to determine how variation in the exposure time and temperature of collagen/GAG membranes to glutaraldehyde vapor affected the density of crosslinking induced. It was found that in the range of 10° C. to 35° C. the mean weight between crosslinks (M_c) reached an equilibrium value after thirty minutes of exposure. The equilibrium M_c value was dependent on the temperature of exposure. It ranged between 24,690±2,650 daltons at 10° C. and 5,420±1,390 daltons at 35° C.

Amino acid analysis was done on both solid state crosslinked collagen/GAG and collagen/GAG membranes crosslinked in aqueous medium, as well as GAG free collgen from different stages in the manufacture of artificial skin. It was found that, of the 15 amino acid residues measured, only the lysine residues were affected by aldehyde treatment. This suggests that solid state crosslinking yields a crosslinked network very similar to that which is produced by crosslinking in aqueous glutaraldehyde.

Recommendations are made for scaling up this technique to large production runs. Suggestions are made for the structure of animal studies to test if the solid state process has any deleterious effects on the medical efficacy of the artificial skin.

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Table of Contents

List of Figures

List of Tables

INTRODUCTION

A) BACKGROUND

As an organ, the human skin is often underestimated in its importance to the proper functioning of the body. The skin serves not only as a barrier to keep out viruses, bacteria, and toxic materials, but also to keep in body fluid and to cushion the other organs from the mechanical shocks and abrasions of daily living. As well, the skin helps to control body temperature, remove wastes, and obtain sensory data. Extensive damage to this organ can be a very serious matter.

Removal of more than 20% (by area) of the skin covering the human body results in a potentially fatal wound.(1) Wounds which result in the removal of both the dermis and the epidermis (full thickness wounds) over more than a few hundred square centimeters are incapable of regenerating new skin. Such wounds close by contracture of the wound area and growth of new skin in from the edges of the wound. Wound contracture around a joint can be severe enough to cripple the victim once scarring is complete.

Full thickness wounds are often treated by grafting. The preferred method for treating large area wounds is autografting. In this method, sections of the patient's own epidermis and the upper portion of the dermis are excised from a donor site and sutured in place over a carefully prepared wound bed. This creates a less serious wound at the donor site so that the more serious wound can be closed.

Obviously, there is a point at which the wound area is so great that autografting results in a very small net benefit for the patient. Meshing the

autograft results in some increase in the area that can be treated. However, cosmetically important areas such as the face and hands still require whole pieces of autograft.

Once all of the appropriate autograft donor sites have been exhausted, surgeons resort to allografting techniques. The two more common techniques are heterografting (using human cadaver skin) and xenografting (most often using processed porcine skin). The major drawback of allografting is that it provides only temporary closure of the wound. There still remains the long process of harvesting skin as it regenerates on the donor sites and grafting it onto the wound sites covered with allografts.

In the last eleven years, an artificial skin has been developed at the Massachusetts Institute of Technology, Fibers and Polymers Laboratory, by Dr. I. V. Yannas and others.(2, 3, 4) In clinical tests, this synthetic skin has proven to be a very good replacement for heterografts. This membrane is composed of an inert outer layer and a biodegradable inner layer.(3, 5)

The outer layer is made from silicone plastic which provides a barrier to toxic substances and foreign organisms as well as regulating the moisture flux through the wound site. The inner layer is made from a coprecipitate of collagen and a glycosaminoglycan(GAG). This material serves as a supporting matrix for migrating dermal cells and thus is the foundation for the neodermis. The collagen/GAG is resorbed as the new dermal layer is formed.

Together, the two layers form a bi-layer composite of both a synthetic and a natural polymer. The silicone layer is discarded once the neodermis has formed. After that, a thin, epidermal autograft is placed on the neodermis and the wound closes up permanently within very few days.

B) MOTIVATION

Clinical results indicate that artificial skin may soon be the treatment of choice for any of a wide variety of large area, full thickness skin wounds. As well, it may see use in reconstructive plastic surgery. The primary motivation for this research is the desire to improve product quality and decrease product cost of what is a very promising medical device.

The present production methods for the manufacture of artificial skin are described in detail elsewhere.(6) Briefly, the process starts with the manufacture of the biodegradable layer. Bovine collagen is dissolved in a low pH solution. The collagen is precipitated out of solution by addition of a glycosaminoglycan. This solution is concentrated to the appropriate density, cast in a thin sheet and lyophilized (freeze-dried).

Freeze-drying yields a thin, open-pored foam. The foam is completely dehydrated by baking in vacuum at 105 degree celcius for twenty-four hours. This dehydrothermal treatment (DHT) imparts a low level of crosslinking. The porous membrane is now strong enough to be coated on one side with silicone and rehydrated in a low pH solution.

If the completed membrane were placed on a patient at this point, the collagen layer would degrade faster than is desirable. Further crosslinking slows down the resorption. This crosslinking is imparted by treatment in a dilute aldehyde solution. The membrane is then rinsed free of the excess aldehyde and stored in 70% aqueous isopropanol to maintain sterility. The skin is rinsed free of alcohol before it is placed on the patient.

There are several disadvantages to the wet crosslinking process. The rehydration, crosslinking and rinse for excess aldehyde takes a total of three days. Collagen slowly degrades in aqueous solution, thus limiting the shelf life of the

membrane. The membrane requires refrigeration. The alcohol storage media places restrictions on how the product is stored and transported.

All of these disadvantages could be remedied if there were some means of imparting crosslinking while the foam remained dry or relatively dry. It has been demonstrated previously that exposing dry foams to vapor from the free surface of 25% w/w aqueous glutaraldehyde induces crosslinking.(7, 8)

C) VAPOR CROSSLINKING

The exact nature of the crosslinks induced in collagen by treatment with glutaraldehyde is not well understood. The crosslinking is known to involve certain amino acid (lysine) side chains on the collagen molecule.(9 - 13) These studies involved collagen crosslinked in aqueous solution. Part of this study is devoted to the amino acid analysis of vapor crosslinked (VXL) collagen. It will be demonstrated that vapor crosslinking affects these same amino groups.

Glutaraldehyde was the only aldehyde investigated in this research. Its use is relatively well understood (see Appendix I) and it is the aldehyde used in the aqueous manufacturing process. Changing aldehydes would necessitate additional testing that was not pertinent to the problem at hand. The long term stability of the reagent in commercial containers wass also tested in this study.

Even vapor crosslinked skins have to be purged of residual aldehyde.(15) Vapor exposure before the DHT step allows the DHT step to serve as the purging step as well. It has been demonstrated that the order of the crosslinking and DHT steps can be reversed with no significant effect on the final level of crosslinking.(14) This research includes studies on membranes crosslinked before DHT and on membranes crosslinked after DHT followed by another 12 hours of DHT. This was done as convenience dictated throughout this research.

The primary focus of this paper is the characterization of the effects of temperature and time of exposure to glutaraldehyde vapor on the crosslink density of the collagen/GAG membrane. It is hoped that these studies will indicate an optimum exposure cycle to duplicate the aqueous crosslinking process now in use.

MATERIALS AND METHODS

A) CONSTRUCTION OF THE VAPOR REACTOR

A constant temperature oven (Model OV-475, Blue M Electric Co., Blue Island, IL) was obtained from the M.I.T. Equipment Exchange. This oven was chosen based on price and its stainless steel construction.

The circulation fan was broken; it was replaced with a muffin fan which was entirely enclosed in the oven, near the top of the chamber . The original heating element and its controller were intended for use in a temperature range well in excess of 40° C. It was replaced with a 500 ohm, ceramic resistor connected to a variable transformer.

The oven was not originally air tight. This was corrected by sealing the internal joints of the chamber with silicone caulking.

Two air vents in the top of the oven were sealed with matched thermometers in rubber stoppers (see Figure 1.). A water dish was glued in place under one of the thermometers and a wick run from the dish to the bulb. Forced circulation from the fan made this arrangement a hygrometer. Thus, both temperature and relative humidity could be measured at the same time.

With the heater off and the fan running, the temperature in the oven holds a steady 33° C. In order to do experiments at lower temperatures, a cooling coil was installed in the lower portion of the chamber near the heater. The coil comprised seven to eight feet of one half inch O.D. vinyl tubing. This was connected directly to the circulating pump of a cooling bath.

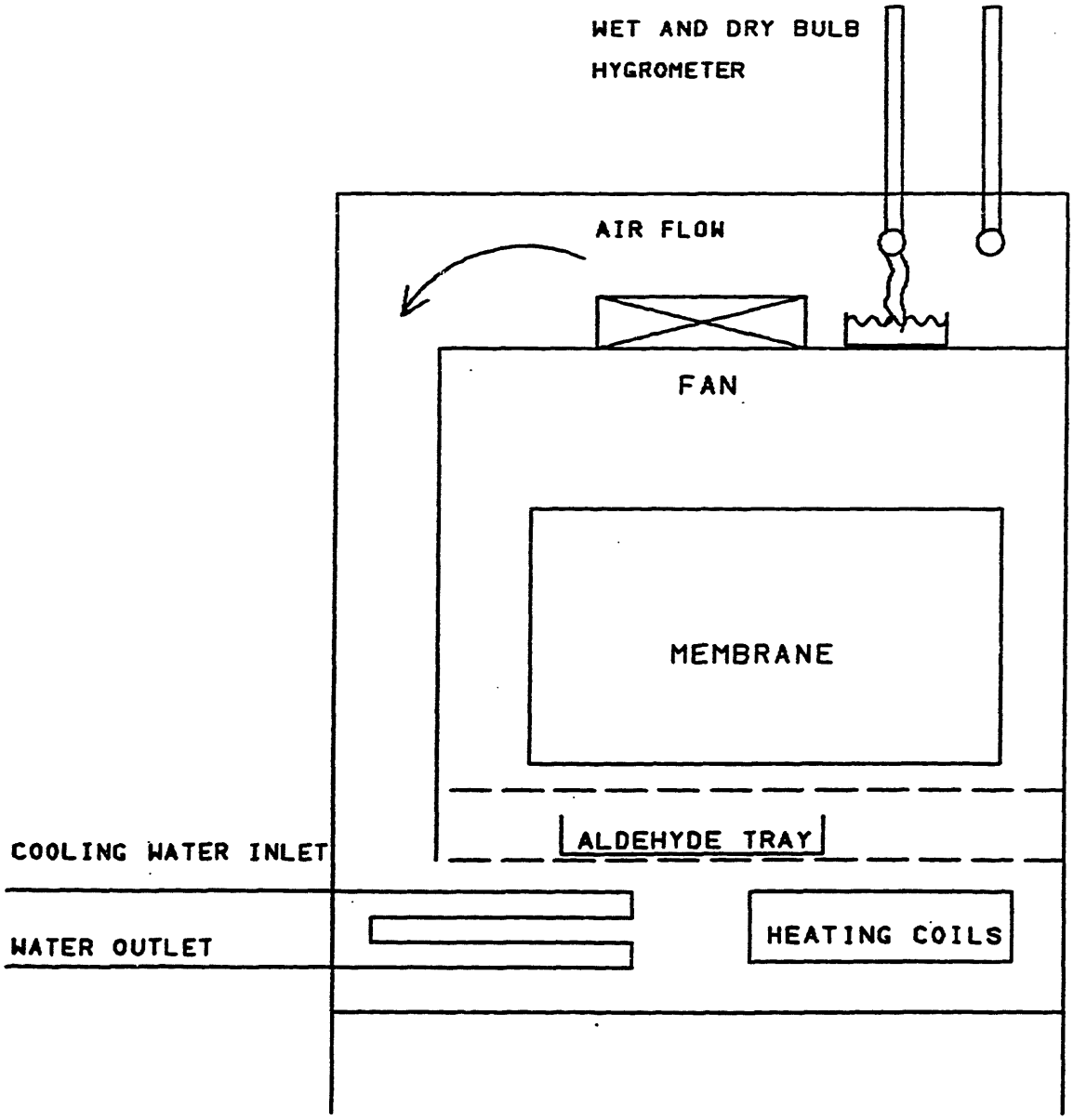

FIGURE 1. Schematic of the vapor reactor. The hygrometer was efficiently stirred by the fan. Air flow over the cooling coil and the heating coils provided uniform temperature in the reactor. This air then passed over the aldehyde tray and the membrane to complete the circulation loop.

The valve on the cooling water system could be used to adjust the temperature coarsely. The heater could then be used to make fine adjustments to the equilibrium temperature.

B) CROSSLINKING PROCEDURES AND REHYDRATION STUDY

All of the membranes tested in this thesis that were vapor crosslinked were done in the reactor described in section A) of this chapter. All of the membranes in this thesis were produced by the protocol up through the DHT step.(6) If the membranes were used as controls, they were wet crosslinked by the protocol in 0.25% w/w glutaraldehyde in pH 3 acetic acid solution. The glutaraldehyde used for all crosslinking, either aqueous or vapor induced, is specified in detail in section E) of this chapter.

The basic vapor crosslinking procedure involved equilibrating the reactor to the desired temperature (either 10° C., 18° C., 24° C. or 35°). The fan was turned on and the water dish for the hygrometer was replenished. During equilibration, one dish containing approximately 30 mL of water and one dish containing approximately 20 mL of glacial acetic acid were in the chamber. These were present to ensure that the humidity in the reactor had also equilibrated.

At least twenty minutes prior to inserting a membrane, a glass dish containing approximately 30 mL of 25% w/w glutaraldehyde was substituted for the water dish. Just prior to being placed in the reactor, the membrane was weighed on an analytical balance. The membrane was then placed in the reactor and left for 5, 15, 30, or 45 minutes. The temperature and the relative humidity were noted at the beginning of the run, the end of the run, and every 15 minutes in between. When the membrane was removed from the chamber, it was weighed again on the balance and then placed in the DHT oven at 105° C. and greater than 25 inches of vacuum. After the DHT step, the membranes were weighed one last time on the analytical balance.

The membranes could be exposed in one of two sequences. It has been shown that the order of the crosslinking and DHT steps does not affect the final crosslink density.(14) Membranes done for the crosslink density versus time and temperature study were exposed after the intial DHT step and then dehydrothermally treated for another twelve hours to rid them of excess aldehyde and to dry them. Membranes prepared for the amino acid analysis detailed in section D) of this chapter were exposed directly out of the lyophilizer and dehydrothermally treated for the normal twenty-four hours.

The total percent weight change of the membrane that was caused by the exposure was determined by subtracting the dry weight after DHT from the weight after exposure. This sum was divided by the dry weight.

To determine the rate at which the membranes picked up moisture, the reactor was equilibrated to 35° C. with only a dish of water in the chamber. A membrane was weighed, placed in the chamber, removed, and weighed. One was done for 1, 3, 5, and 10 minutes each. Each was dehyrothermally treated for twelve hours and weighed when it was removed. This procedure was repeated with a dish of acetic acid present. This was done to determine what effect the acetic acid vapor had on the rate of rehydration.

Finally, a study was done to determine if a preliminary period of rehydration would affect the equilibrium value of the crosslink density. The reactor was equilibrated to 24° C. with water and acetic acid present in the chamber. One membrane was placed in the chamber for twenty minutes. At this time, a dish containing approximately 30 mL of glutaraldehyde and another membrane were placed in the reactor. The membranes were left for one hour to ensure that the aldehyde vapor had time to equilibrate. The membranes were weighed when they were removed and then subjected to the DHT step. They were weighed again when removed from the oven. The percent weight change was calculated as before.

C) DETERMINATION OF CROSSLINK DENSITY

The efficacy of crosslinking procedures for collagen/GAG materials is measured in vitro. There are chemical means of determining the presence of crosslinking. One of these methods is described in section D) of this chapter. The mechanical test described in this chapter is much easier and less time consuming.

The extent of crosslinking in the collagen/GAG membranes is determined by a constant strain, stress relaxation test. The membranes are denatured to remove naturally occurring crosslinks. The denatured membrane is considered an ideal rubber; as such,the stress relaxation test yields information which correlates directly to the mean weight between crosslinks or M_c.

The procedure is described in detail elsewhere.(16, 17) In brief, it involves cutting samples of the membrane approximately 2.5" x 0.5", typically three to five specimens per sample. Either a pair of scissors or a sharp razor blade is used to get straight edges without nicks. These strips are immersed individually in 80° C., 0.9% w/v saline solution. Each strip is allowed to denature for at least five minutes.

Each strip is then mounted in a set of jaws attached to a 500 gram load cell in an Instron mechanical testing machine (tabletop model 1122, Instron Co., Canton, MA 02021). The jaws are immersed into the 80° C. saline solution and the test proceeds.

The sample is extended to 5% strain on a one inch gage length. It is allowed to relax for four minutes. It is strained to 10% strain and allowed to relax. Then, it is strained to 15% strain and allowed to relax. Finally it is returned to zero strain.

The width of the sample is measured with a ruler, the thickness with a pressure sensitive micrometer (100 gram anvil load). The stress is calculated on this crosssectional area.

Since the membrane is a porous structure, the volume fraction must be measured. This is done by measuring the wet and dry weights of the test specimen and taking a ratio of their values.

The mean weight between crosslinks, M_c measured in daltons is calculated by regressing the equilibrium stress at a given strain against that strain to get a slope. Regressions were done with a regression program on a Hewlett-Packard HP-29C handheld calculator (Hewlett-Packard, Corvallis, OR 97330). Individual specimens which regressed with a correlation factor of less than .96 were discounted as invalid. The slope was then divided into the volume fraction raised to the one third power and the total multiplied by a constant.

D) AMINO ACID ANALYSIS OF CROSSLINKED COLLAGEN/GAG

It has not been previously determined whether aqueous and vapor induced crosslinking in collagen/GAG are similar in nature. Amino acid analysis was done on collagen taken from various stages of the manufacturing process. These samples were analyzed for fifteen different amino acids (listed in Appendix II). Glycine was used to normalize the different runs with 324 residues of glycine per 1000 residues as the standard value.

The samples described above were prepared for analysis and analyzed by Mr. Scott Berceli. In brief, this involved hydrolysis of about 0.1 gr (acurately weighed) of each dry sample at 110° C. with 5 mL of 6N HCl in a vacuum sealed ampoule. The samples were thoroughly degassed by alternated heating and freezing under vacuum (three cycles) prior to sealing.

The hydrolysis was terminated at twenty-two hours for each sample. The hydrolyzate was neutralized in Spritz Neutralization Buffer.(18) The procedure is described in detail elsewhere.(18, 19) Once the hydrolyzate had been mixed with the appropriate reagents, it was loaded into the column of a Model 119B Beckman amino acid analyzer (Spinco Div., Beckman Instruments, Palo Alto, CA).(20) The pertinent operating conditions are as follows:

Resin:	W-2
Resin Bed Geometry:	300 mm X 9 mm
Temperatures and Change Time:	50° C. at start set to 65° C. at 30 min.
Flow Rates:	Buffer, 70 mL/hr Ninhydrin, 35 mL/hr
Buffers (and Beckman cat.#):	a) pH 2.94, 0.2N Na+, (PN-335342) b) pH 4.10, 0.2N Na+, (PN-338587) c) pH 7.32, 0.2N Na+, (PN-338588) d) 0.2N NaOH
Buffer Changes:	Change buffer a) to b) at 100 min. Change buffer b) to c) at 119 min. Change buffer c) to d) at 195 min. NaOH to equilibrate at 200 min. and equilibrate for 25 min.
Total Cycle Time:	225 min.
Recycle Module:	Set to 'Single Column'

There were seven different samples tested in the amino acid analysis. Four of them were vapor crosslinked samples and three of them were controls. Two aliquots were run for each sample. The results reported are the average of those two runs.

The first control was bovine hide collagen derived from USDA delimed cow hide. Collagen from an identical source is used in the manufacture of artificial skin. The second control was porous membrane taken just after the DHT step in the manufacturing process. The collagen in this sample had been dispersed and swollen in acetic acid solution; precipitated with a glycoaminoglycan (GAG); lyophilized; and heated in vacuum at 105° C. for twenty-four hours. The last control was an uncoated piece of artificial skin. The collagen in this sample had been rehydrated; crosslinked in a 0.25% w/w aqueous glutaraldehyde solution; dialyzed in water for twenty-four hours; and relyophilized.

The four vapor crosslinked samples were produced generally as described in section B) of this chapter. The porous membranes were taken directly from the freeze-drier and exposed at 34°±2° C. and 87-90% relative humidity. One sample was exposed for 1, 1.5, 2, and 3 hours respectively. After exposure, the samples were dehyrothermally treated for twenty-four hours.

All of the sample for the amino acid analysis were stored in a desiccator until testing by Mr. Berceli. The M_c was measured on all of the controls and on the vapor crosslinked samples by the procedure outlined in section C) of this chapter. To do a crosslink density measurement on the native bovine collagen, a porous membrane was prepared by the protocol and lyophilized with the exception that no GAG was added during the blending process. The porous membrane was tested right after lyophilization.

E) GLUTARALDEHYDE STABILITY STUDY

In order to test how the activity of the glutaraldehyde reagent decayed with time under various conditions, an aldehyde sensitive assay was done on the glutaraldehyde right after the bottle was opened and again after 237 days (approximately 2/3 years).

The aldehyde assay is one developed for use with Purpald (Aldrich #16,289-2, Purpald Gold Label, Aldrich Chemical Co., Milwaukee, WI 53201). The assay is done as follows:

1) prepare Purpald solution by mixing 15 gr of Purpald per liter of 1M NaOH,

2) mix 1 mL of Purpald solution with 3 mL of test solution in a test tube and vortex for 10 seconds on low setting,

3) place test tube in rack on agitating table and agitate for 30 minutes on medium setting,

4) zero spectrophotometer (Coleman Model 6/20A, Coleman Instruments, Maywood, IL) and then set 100% transmittance at 552 mu on a water and Purpald solution blank,

5) then read the transmittance of the test solutions.

The test solutions were serial dilutions of the glutaraldehyde taken from the bottle when it was first opened, so there was no control or reference solution for this study. The glutaraldehyde tested was the same 25% w/w aqueous glutaraldehyde used for all of the crosslinking runs done in this thesis (Baker Analysed Reagent, #2127-1, J.T. Baker Chemical Co., Phillipsburg, NJ 08865).

For the first assay, 4 mL of aldehyde was frozen for twenty- four hours. It was thawed at room temperature and tested along with aldehyde taken directly

from the bottle. In both cases, a serial dilution was made of the aldehyde such that 0.25% w/w, 0.025% w/w, and 0.0025% w/w solutions of both fresh and of frozen aldehyde were tested. Frozen glutaraldehyde was tested to determine if freezing was a viable means of increasing the shelf life of the reagent.

For the second assay, 237 days later, 4 mL of aldehyde which had remained frozen was thawed to room temperature and was compared with aldehyde which had remained in the bottle. In the latter case, the bottle had been stored away from light and at room temperature. Unlike the frozen sample, the bottle had been opened roughly twenty different times to obtain aldehyde for the crosslinking runs. As with the first assay, serial dilution of both the frozen and the plain aldehyde. These solutions were tested by the assay listed above.

F) STERILIZATION STUDY

In order to test the sterilizing capability of exposure to glutaraldehyde vapor, several spore strips were exposed to gaseous glutaraldehyde for various lengths of time. The reactor was prepared as described in section B) of this chapter. The temperature for the run was 34° C. Approximately 30 mL of 25% w/w glutaraldehyde (J.T. Baker Chemical Co.) was placed in the reactor at the start of the run.

The spore strips used in this experiment were for use in testing the efficacy of gas or dry heat sterilization. Each strip contained 3.98×10^6 colony forming units of *Bacillus subtilis var. niger* (Raven Bio-Lab, Inc., Omaha , Nebraska 68106).

Five spore strips were all placed in the reactor at the start of the run. One strip was removed at 30, 45, 60, 90, and 120 minutes respectively. The relative humidity was measured at the time that the individual strip was removed.

When the run was completed, the five test samples and an unexposed control sample were submitted to the Division of Comparative Medicine at MIT for culturing and evalutation. The culturing involved incubation for 7 days at 37° C. in Trypticase Soy Broth. Gross observation of the culture was sufficient to determine whether any spores had survived the exposure to aldehyde vapor (positive result). No controls for negative result were run in this study.

RESULTS AND DISSCUSSION

A) GENERAL OBSERVATIONS

The modified oven worked very well as a test reactor. It was simple to equilibrate. It took on the order of two hours to bring the reactor from room temperature to either temperature extreme. Once the valve setting for the cooling water and the transformer setting for the heater were determined for a given temperature, the reactor could be brought back to that temperature reliably.

The hygrometer was sufficiently sensitive to detect when the door to the reactor had been opened for periods as short as ten seconds. With the door closed, there was no noticable odor of glutaraldehyde.

The membranes produced by the reactor came through the exposure with little detectable damage. During one of the early trials, the corner of one membrane became tangled in the fan. That membrane was discarded and a rack was installed in front of the fan to prevent future entanglements.

Some of the membranes became slightly yellow during the vapor exposure and this yellowness became darker during the DHT step. The yellowness occurred with membranes which had been exposed at 35° C. for periods longer than thirty minutes. It did not occur with membranes exposed for shorter periods or at lower temperatures for any period measured.

B) EXTENT OF REHYDRATION OF COLLAGEN/GAG MEMBRANES AFTER EXPOSURE TO VARIOUS MEDIA

There are three compounds in the vapor reactor which affect the crosslinking of the membranes. These are water vapor, acetic acid vapor, and glutaraldehyde vapor. Membranes were also exposed to water vapor alone and a combination of water and acetic acid vapors. This was done to get a rough estimate of the time required for the membranes to absorb as much vapor as they capable of absorbing. The membranes take in excess of ten minutes to rehydrate completely in the presence of moist air. The results shown in Table 1. seem to indicate that the presence of acetic acid vapor makes some difference on the rate and the amount of moisture picked up by the membrane. This stands to reason, as collagen is known to swell in low pH solution.(21) As the acetic acid vapor is absorbed by the collagen/GAG membrane, it would cause the material to swell more and absorb more than the material which was exposed only to water vapor. Only one specimen was weighed at each data point in Table 1.; thus, no standard deviations are stated and the statistical signifigance of the presence of acetic acid vapor cannot be determined.

TABLE 1. RESULTS OF THE REHYDRATION STUDY

	Percent Weight Moisture After Minutes of Exposure			
Conditions	1	3	5	10
35° C. w/o acetic acid	14.1%	16.8%	17.4%	18.5%
35° C. with acetic acid	17.1%	18.3%	23.8%	23.1%

The maximum weight gain for exposure to water and acetic acid vapor appears to be on the order of 23% w/w. However this still requires five minutes to achieve. The results shown in Table 2. show that this can be achieved in much less than five minutes when glutaraldehyde vapor is present. Here, a total percent weight change on the order of 32% w/w was reached by five minutes of exposure at 35° C.

TABLE 2. PERCENT WEIGHT CHANGE DURING VAPOR EXPOSURE

Temperature	Total Percent Weight Moisture After Minutes of Exposure			
	5	15	30	45
10° C.(a)	13.0%	18.2%	19.5%	18.3%
18° C.(b)	21.6% ±4.6%	22.9% ±7.4%	23.2% ±7.3%	24.3% ±4.7%
24° C.(c)	26.3% ±4.9%	26.2% ±4.2%	25.2% ±3.1%	28.0% ±4.4%
35° C.(a)	31.3%	33.3%	33.3%	33.5%

a) Only one sample done at each time.
b) Mean of two samples, plus and minus one standard deviation.
c) Mean of three samples, plus and minus one standard deviation.

Table 2. shows an increase in total percent weight change with an increase in temperature. Also, the equilibrium value for the total weight change is much higher when glutaraldehyde is present than when it is not. The membranes appear to have reached their maximum weight change in five minutes or less independent of temperature. This indicates that the glutaraldehyde promotes the absorption process in collagen/GAG. Further testing would have to be done to determine whether the glutaraldehyde the collagen fibers to swell or if the greater absorption is caused by another mechanism.(12, 13)

C) M_c RESULTS

There were a total of seven time and temperature variation runs done in the reactor. One run was done at 10° C. and 35° C. respectively. Two runs were done at 18° C. and three runs were done at 24° C. When multiple runs were done at a given temperature, these were done on separate days to validate the reproducibility of the data.

The results of the crosslink density tests on the membranes produced in these runs are presented in Figures 2. through 5. Three M_c specimens were done on each membrane. The results from multiple runs were averaged together for the graphs shown in the figures.

Each graph plots the mean weight between crosslinks in daltons against the time at which it occurred. The error bars indicate plus and minus one standard deviation. Figure 6. shows a composite of the four individual graphs. It can be seen in this figure, that the equilibrium M_c is reached in fewer than thirty minutes of exposure even in the 10° C. case. Furthermore, at the higher temperatures, a majority of the crosslinks have been induced by five minutes of exposure.

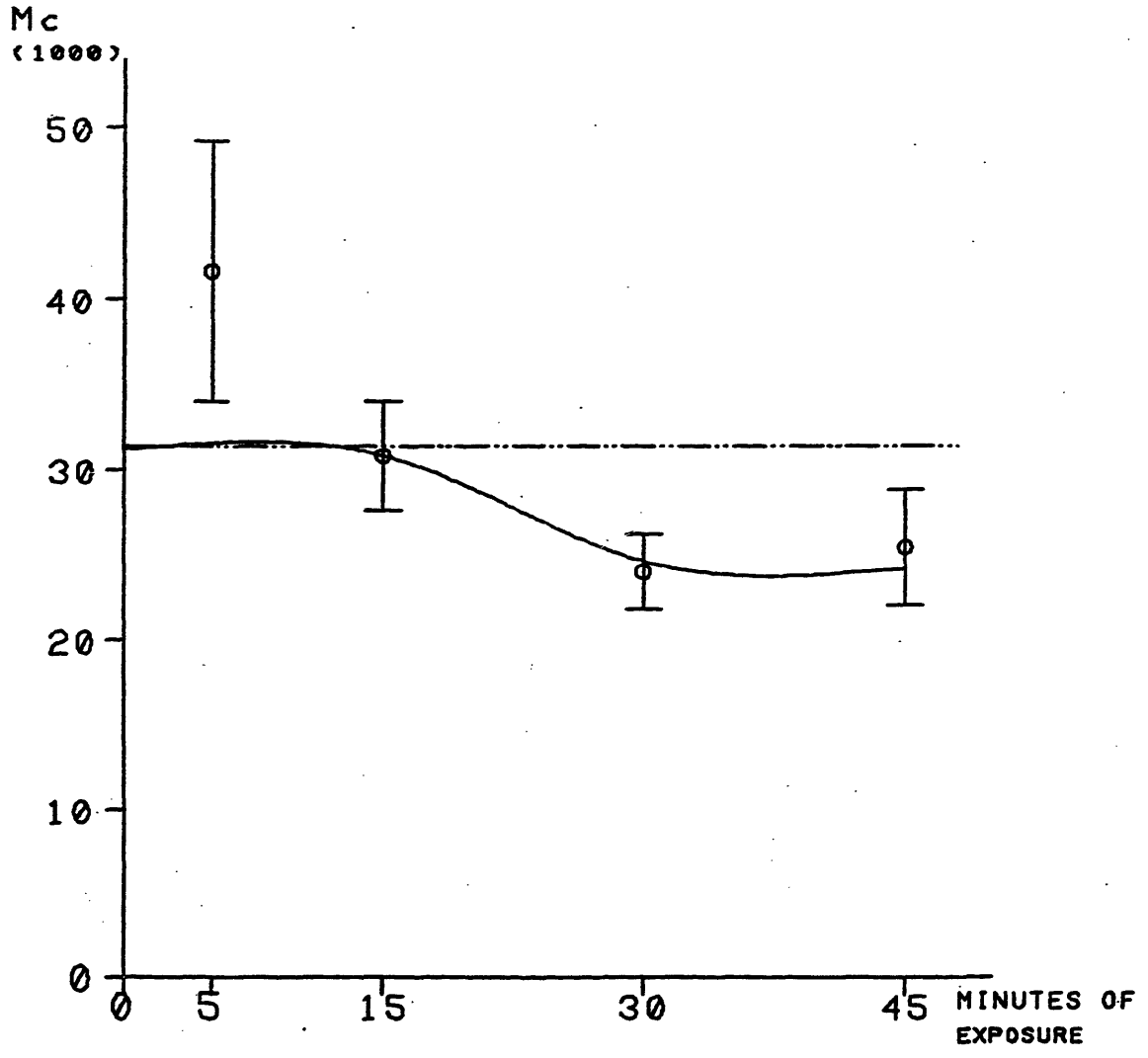

FIGURE 2. Mean weight between crosslinks versus time of exposure to aldehyde vapor at 10° C. at 70% relative humidity. M_c values plotted ± one standard deviation (N = 3).

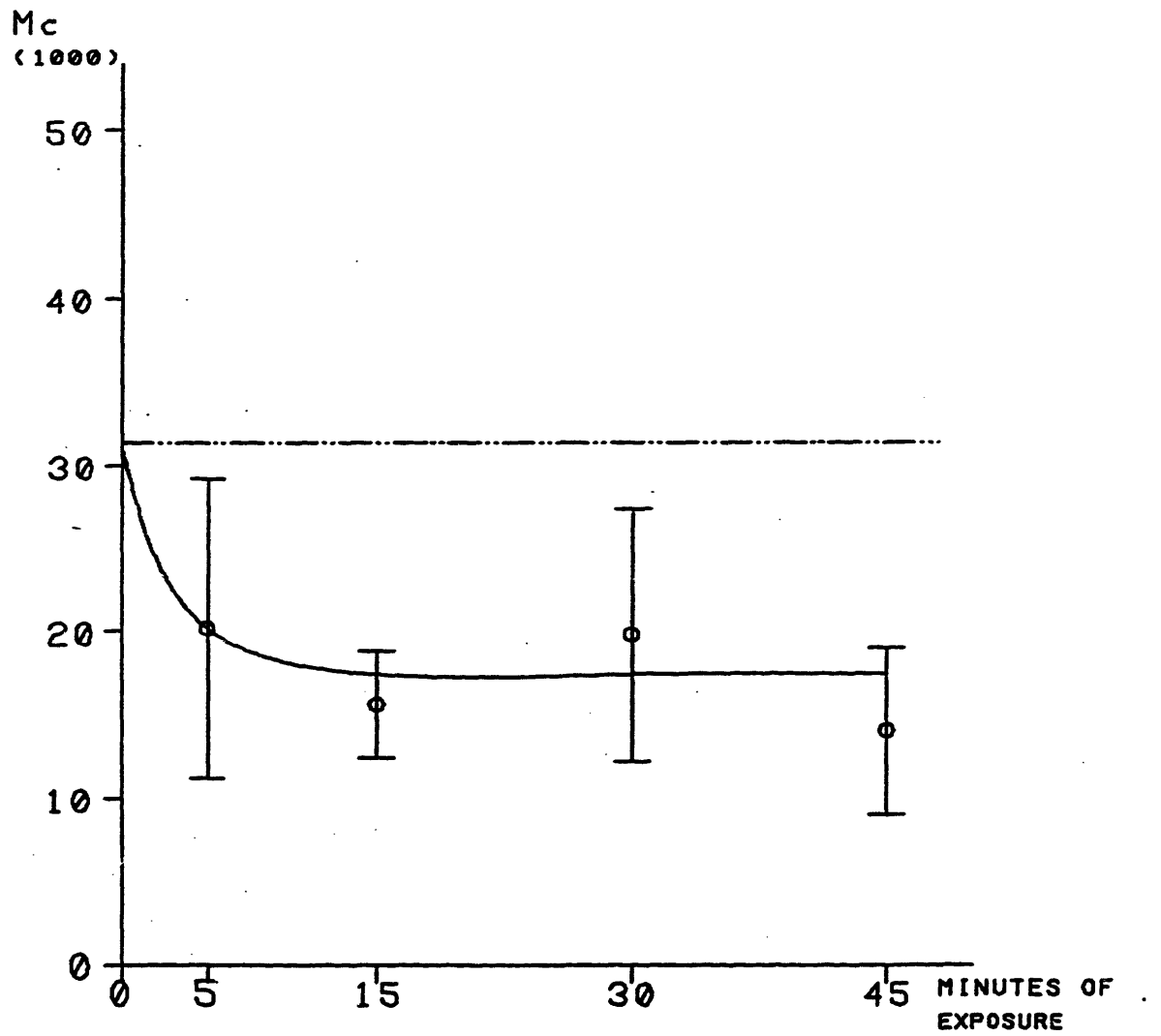

FIGURE 3. Mean weight between crosslinks versus time of exposure to aldehyde vapor at 18° C. at 75% relative humidity. M_c values plotted ± one standard deviation (N = 6).

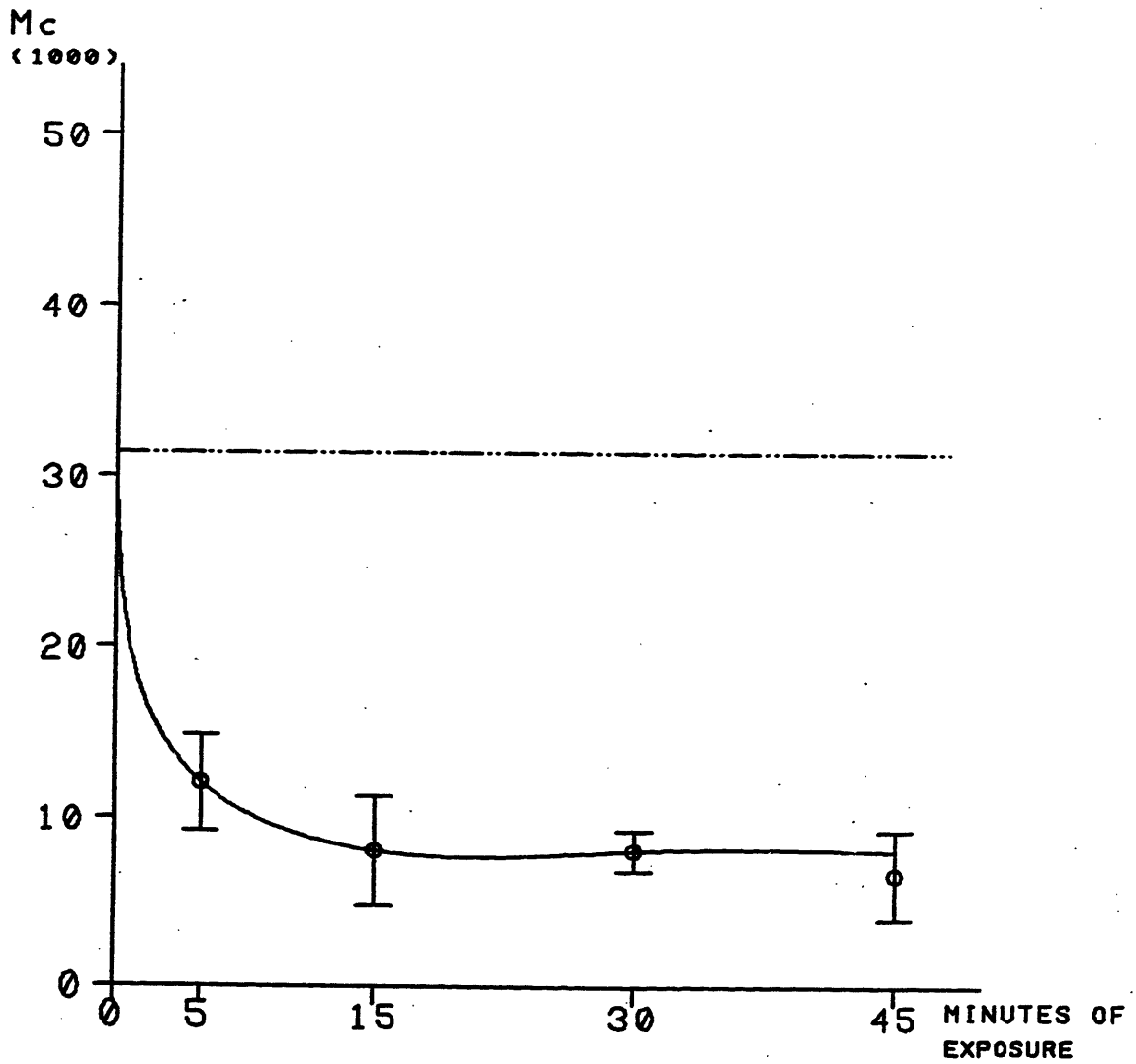

FIGURE 4. Mean weight between crosslinks versus time of exposure to aldehyde vapor at 24° C. at 75% to 80% relative humidity. M_c values plotted ± one standard deviation (N = 9).

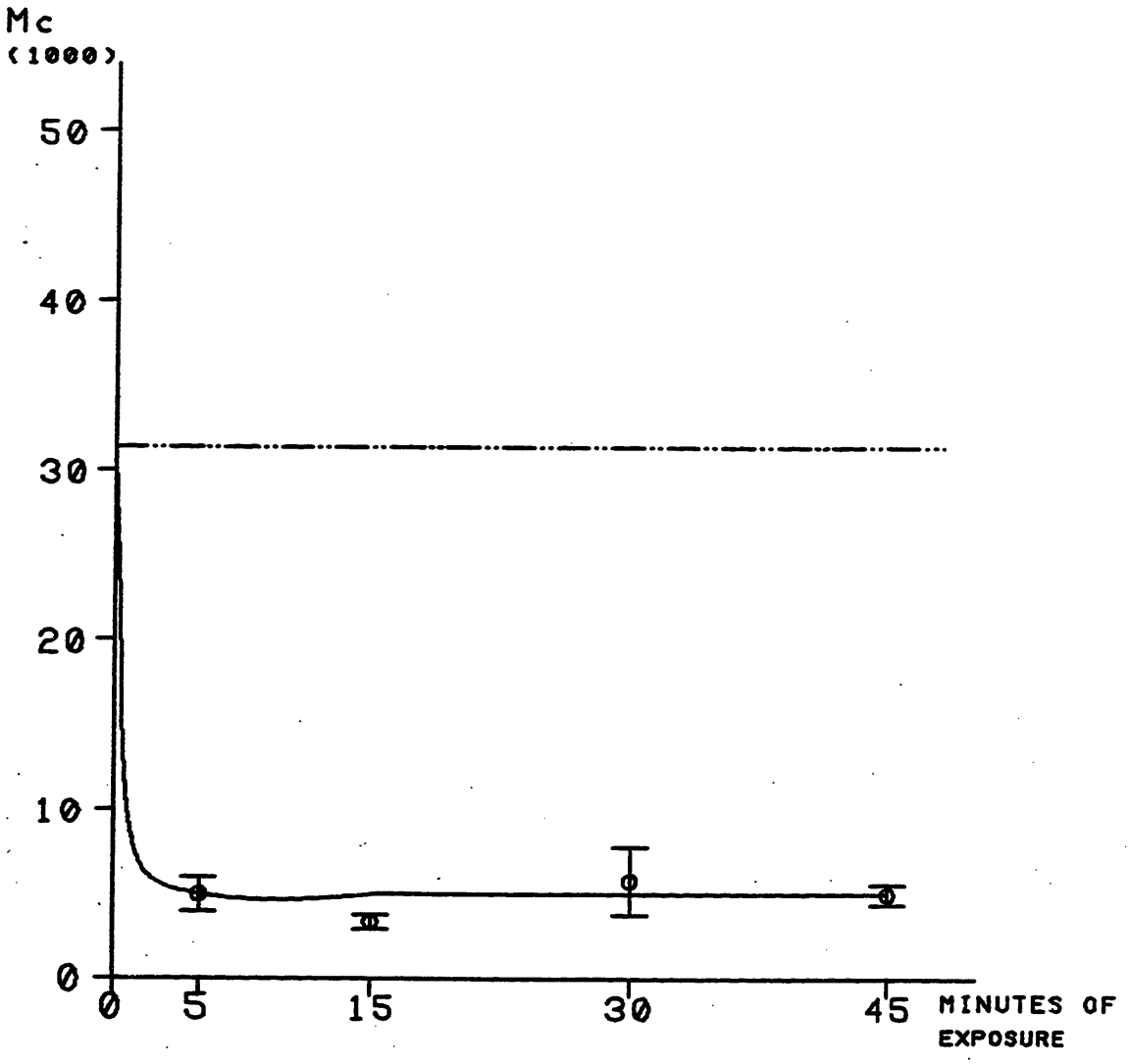

FIGURE 5. Mean weight between crosslinks versus time of exposure to aldehyde vapor at 35° C. at 93% relative humidity. M_c values plotted ± one standard deviation (N = 3).

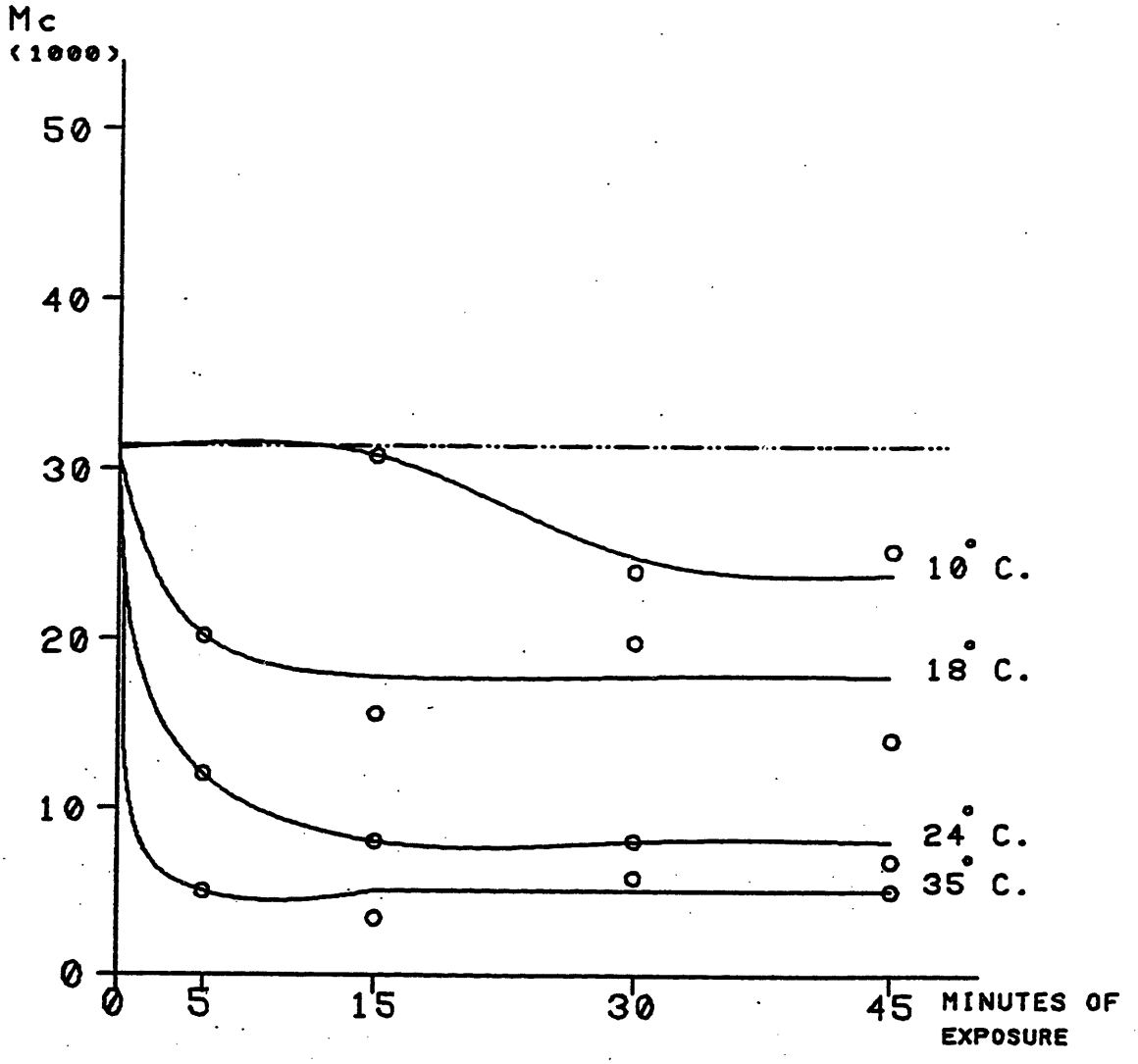

FIGURE 6. Composite plot of M_e curves by temperature versus time of exposure to aldehyde vapor. In the range of 10° C. to 35° C., the equilibrium value M_e is reached in fewer than thirty minutes of exposure.

Figure 7. contains a plot of the equilibrium M_c versus the exposure temperature. The equilibrium M_c was calculated by taking the average of the results for all specimens exposed for thirty and forty-five minutes. Again, the error bars indicate plus and minus one standard deviation. The material which is crosslinked in the aqueous phase typically has an M_c between 10,000 and 14,000 daltons.(8) The solid state crosslinking temperature to produce an equivalent level of M_c is near 20° C., as seen in Figure 7.

The preliminary rehydration of the membrane in water and acetic acid vapor does not appear to have had any effect on the equilibrium M_c. Table 3. shows the M_c and the total percent weight gain for the membranes used in the preliminary rehydration study. The values on the left are for the membrane which was exposed to water and acetic acid vapor for twenty minutes prior to the introduction of glutaraldehyde vapor. The values on the right are for the membrane which was placed in the reactor at the same time as the dish of glutaraldehyde. The entire membrane was weighed in each case, no standard deviation is given for the total percent weight change.

TABLE 3. EFFECT OF PRELIMINARY REHYDRATION ON EQUILIBRIUM CROSSLINK DENSITY(a)

	20 Minute Rehydration	Dry Membrane
Total Percent Weight Change	23.4%	27.7%
Equilibrium M_c	6,750±490	5,570±1,240

a) All specimens were crosslinked at 24° C. for 1 hour.

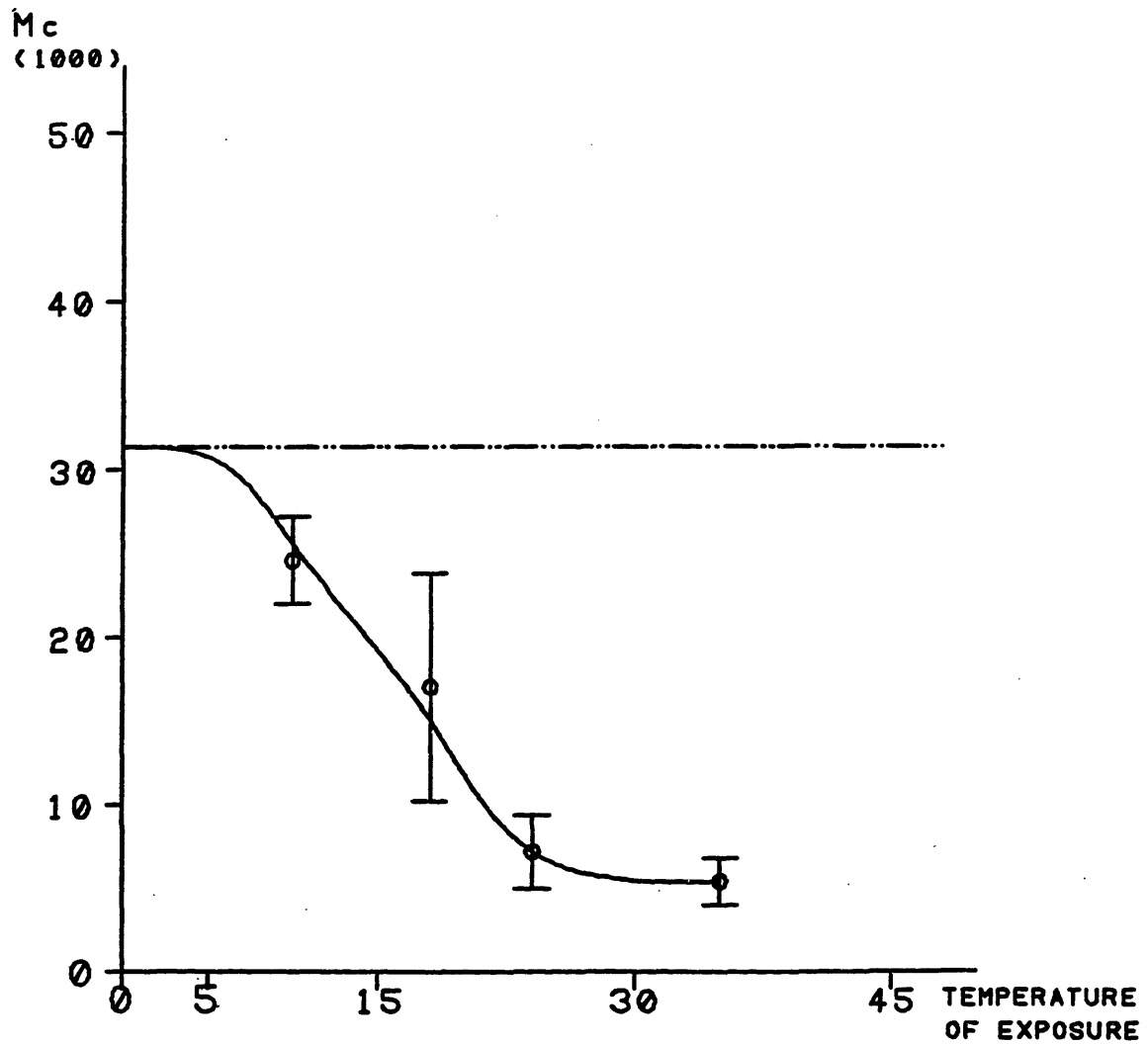

FIGURE 7. Equilibrium value of M_c versus the temperature of exposure. For each temperature, the M_c is the average of specimens exposed for thirty and forty-five minutes (N = 6, 12, 18, and 6 for temperature = 10° C., 18° C., 24° C., and 35° C. respectively). M_c values plotted ± one standard deviation.

The data presented in Table 3. are consistent with data for 24° C. presented in Table 2. and Figure 4. This indicates that although a membrane was exposed to a preliminary period of rehydration in water and acetic acid vapor, the glutaraldehyde vapor was, nevertheless, capable of diffusing into the collagen/GAG and inducing crosslinks.

D) AMINO ACID ANALYSIS

Fifteen amino acids were determined in each sample (complete results are given in Appendix II). Of these, only the lysine residues showed any signifcant variation with exposure to glutaraldehyde. Thus, only the lysine residues per thousand residues are shown in Table 4. (the average of two runs). In the left hand column, the samples are described briefly. Their exposure time is listed as are the mean densities between crosslinks plus and minus one standard deviation (as determined by the process outlined in section C) of the Materials and Methods chapter).

There are two things which should be noted about this table. First, all of the vapor exposures were done at 35° C. and approximately 90% relative humidity. These membranes were exposed prior to the DHT step. As can be seen by comparing the M_c results given in the table with the results shown for 35° C. exposure in section C) of this chapter, there is no significant difference in crosslink density.

The second thing to be noted is that the M_c value for the standard membrane that was crosslinked in aqueous solution is uncharacteristically low. Mean densities between crosslinks of between 10,000 and 14,000 for aqueously crosslinked material are more common. However, the lysine residue value stated is taken to be indicative of the M_c stated. One possible explanation for the low M_c is that the room temperature may have been higher than normal when the material was aqueously crosslinked.

TABLE 4. RESULTS OF THE AMINO ACID ANALYSIS, LYSINE RESIDUES PER THOUSAND RESIDUES

Sample Description	Exposure Time	Crosslink Density	Lysine Residues
Native Bovine Collagen	0 hr	45740±13260(a)	30.4
Protocol Membrane through DHT	0 hr	31440± 7520(a)	28.4
Protocol Membrane Wet Crosslinked	24 hrs	6600± 1230	19.3
Vapor Crosslinked	1 hr	3220± 1260	5.4
Vapor Crosslinked	1.5 hrs	(b)	5.0
Vapor Crosslinked	2 hrs	(b)	3.9
Vapor Crosslinked	3 hrs	(b)	5.3

a) M_c tests were not done on the samples submitted for amino acid analysis, rather on similar samples.
b) M_c tests were not done on these samples, their M_c value should be equal to or lower than the value for one hour exposure.

In any event, it can be seen readily that the available lysine residues decrease dramatically with exposure to gaseous aldehyde. Even as brief an exposure as one hour at 35° C. is sufficient to reduce the available lysine residues to one third the value found with 24 hours of aqueous exposure (at room temperature).

The curve in Figure 8. shows that the mean weight between crosslinks decreases rapidly with the initial decrease in available lysine residues (from 30 to 20 residues per 1000). This is followed by a relatively slight decrease in M_c with a decrease in lysine residues from 20 to 10 residues per 1000. Unfortunately, no samples of collagen/GAG that were crosslinked in gaseous glutaraldehyde exhibited the same M_c as the aqueously crosslinked collagen/GAG. This would have allowed a direct comparison of lysine residues.

E) STABILITY STUDY

The results of the stability study are presented in Table 5. Over the period of 2/3 years, there was no degradation in the potency of the glutaraldehyde as measured by this assay. The freezing of the aldehyde did not adversely affect its potency. However, for periods up to eight months, freezing is an unnecessary precaution to extend the shelf life of the reagent.

The table shows the transmittance at 552 nm of various serial dilutions of fresh and frozen aldehyde. Under each type, the column on the left is for one day after openning the bottle and the column on the right is for 237 days after openning.

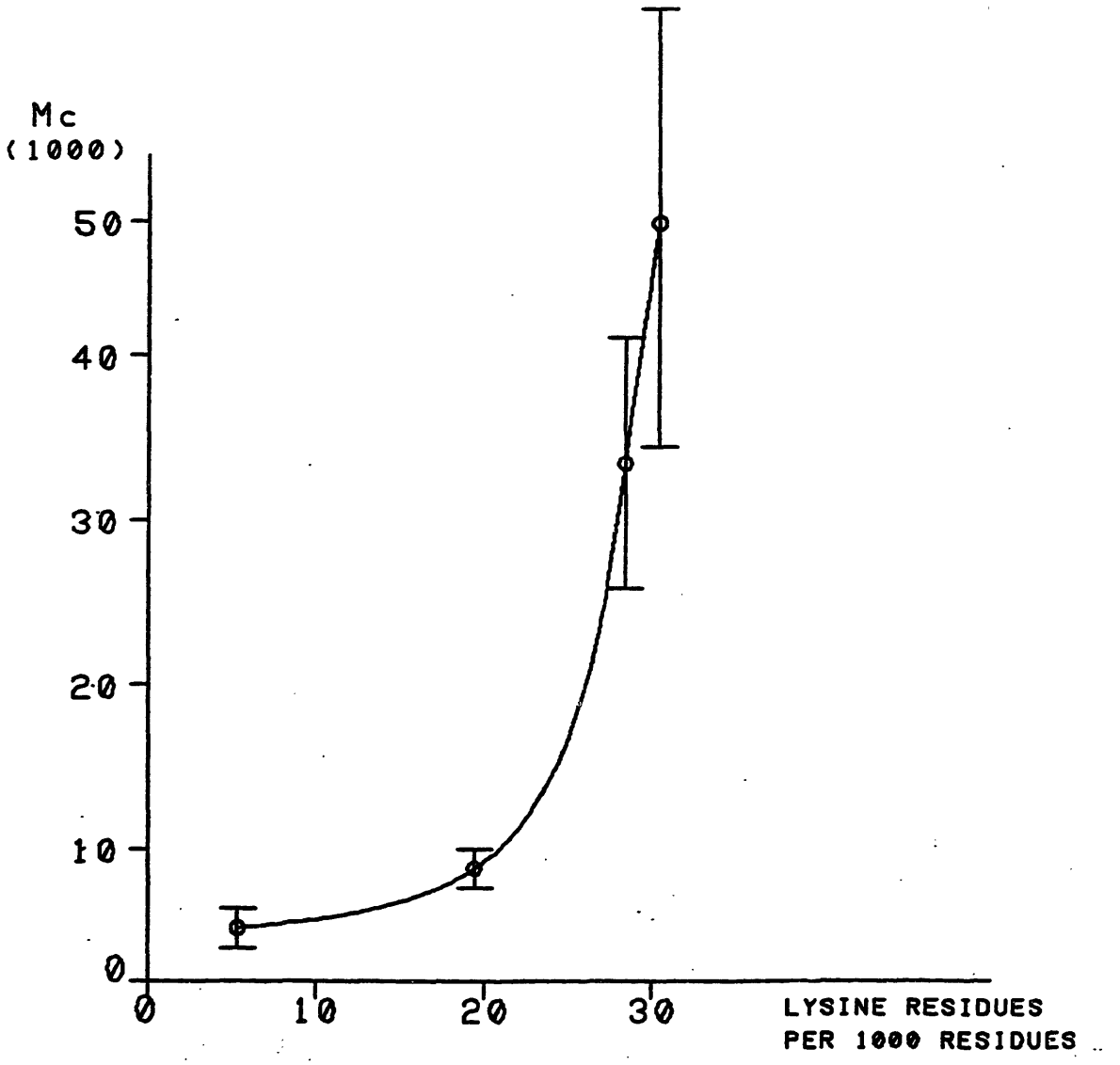

FIGURE 8. Mean weight between crosslinks versus lysine residues per thousand amino acid residues. The M_c values plotted ± one standard deviation ($N \geq 4$). From left to right on the graph: 1) vapor crosslinked one hour at 35° C.; 2) wet crosslinked for twenty-four hours at room temperature; 3) uncrosslinked membrane processed through the DHT step; and 4) native bovine collagen.

TABLE 5. RESULTS OF THE PURPALD ASSAY ON GLUTARALDEHYDE

Strength of Solution	Percent Transmittance at 552 nm of			
	Fresh		Frozen	
(% w/w)	0 days	237 days	0 days	237 days
0.25%	0.5%	0.5%	0.5%	0.8%
0.025%	5.0%	5.8%	6.0%	4.8%
0.0025%	80.0%	79.0%	79.0%	79.0%

F) STERILIZATION STUDY

The results of the sterilization study are shown in Table 6. As can be seen, all of the spore strips tested positive for viable spores. This indicates that exposure to glutaraldehyde vapor for up to two hours at 34° C. and 31% relative humidity is insufficient to sterilize a sample. Sample number 1 is the control spore strip that was not exposed to any aldehyde vapor.

TABLE 6. RESULTS OF THE STERILIZATION STUDY

Sample Number	Time of Exposure	% Relative Humidity	Culture Results
1	0 minutes	22% (a)	Positive
2	30 minutes	32% (b)	Positive
3	45 minutes	31% (b)	Positive
4	60 minutes	30% (b)	Positive
5	90 minutes	31% (b)	Positive
6	120 minutes	30% (b)	Positive

a) relative humidity in room on day of test.
b) exposed to gaseous glutaraldehyde at 34° C.

The relative humidity that is typically found in ethylene oxide sterilization is on the order of 45%. The low relative humidity of the glutaraldehyde study may have affected the results. As noted in the figures in section C) of this chapter, relative humidities in excess of 90% are attainable. It could be that exposure for some period of time at 34° C. and greater than 90% relative humidity would sterilize the sample.

CONCLUSIONS AND RECOMMENDATIONS

The results from both the amino acid analysis and the mechanical crosslink density tests demonstrate beyond any reasonable doubt that the exposure of collagen/GAG membranes to glutaraldehyde vapor induces crosslinking. The results of the amino acid analysis strongly indicate that there is great similarity between crosslinks induced by gaseous glutaraldehyde and crosslinks induced by aqueous treatment as in the present product.

The equilibrium level of vapor induced crosslinking can be altered by adjusting the exposure temperature and exposing the membrane for more than thirty minutes. There are, however, definite limits to this control, as explained below.

Exposure temperatures much lower than 0° C. will induce no crosslinking in conveient time scales as the glutaraldehyde will have frozen (for 25% w/w aqueous glutaraldehyde, $T_M = -7° \pm 0.5°$ C.). Likewise, exposure temperatures above 40° C. induce crosslinking. Yet exposure at such temperature and humidity also denatures part or all of the collagen. This temperature range corresponds to a mean weight between crosslinks range of roughly 50,000 daltons at the low temperature end to 3,000 daltons at the high temperature end.

At present, aqueously crosslinked artificial skin is produced with a mean weight between crosslinks of 10,000 to 14,000 daltons. This indicates an equivalent solid state crosslinking temperature of 20° C. As one would expect, this is very close to room temperature, the temperature of aqueous crosslinking.

The first recommendation indicated by this research is the investigation of vapor exposure of the membranes during the last stages of the lyophilization

process. The crosslink density is independent of the order in which the membranes are crosslinked and subjected to the DHT step. Thus, it would make sense to expose the membranes while they are in a gas tight chamber and still in the pans on the shelves.

The glutaraldehyde vapor could be introduced through the chamber vent-to-atmosphere. A fan would have to be installed in the vacuum chamber to insure adequate circulation of the glutaraldehyde, water, and acetic acid vapor. The shelves would provide excellent thermal control during the crosslinking phase. Humidity can be control with extreme reproducibility because both the chamber volume and the volume of glutaraldehyde added to it would be fixed.

Once the crosslinking prcedure had been completed, the vacuum pumps would be reconnected to remove unused vapor. The shelf temperature would be raised to aid in driving off the last of absorbed the moisture and the aldehyde. The membranes would then subjected to the DHT step, coated, and packaged in dry nitrogen.

The aldehyde vapor would be trapped in the condenser. When the condenser was flushed, care would have to be taken to ensure that the glutaraldehyde was diluted sufficiently.

The second recommendation involves the sterilization capability of glutaraldehyde. Solutions of glutaraldehyde are routinely used to disinfect respirators and similar surgical equipment. The undesirable results of the sterilization study may have been caused by the low relative humidity. The study should be repeated at higher humidity to determine whether the higher humidity increases the efficacy of the process.

A problem could arise from the effect of temperature on the crosslinking process. As mentioned above, the most desirable croslinking temperature is 20° C. while the most desirable sterilization temperature would most likely be between 37° C. and 40° C. Still, the experiment should be tried. The crosslinking reaches an equilibrium level related to tempenerature of exposure, so it would be possible to expose the membrane for as long as is necessary to acheive sterilization.

The last set of recommendations has to do with animal studies. Before artificial skin produced by vapor crosslinking can be released for clinical trials on human patients, there would have to be exhaustive animal studies done to determine if the change in processing adversely affected the performance of the material.

Membranes produced in this reactor were used in two separate animal studies done by Ms. Elaine Lee.(22) The particulars are given in Table 7. The control in each study was a membrane that was aqueously crosslinked. The VXL membrane in the first study was exposed to aldehyde vapor for 60 minutes at 35° C. without acetic acid vapor present. The VXL membrane in the second study was exposed to aldehyde vapor for 15 minutes at 35° C. with acetic acid vapor present. The values given are stated plus and minus one standard deviation. The M_c values for the VXL and the protocol control in the first study are both uncharacteriscally high.

The D_{50} is a measure of the contraction rate of the wound. The value stated is the number of days after surgery at which the wound has contracted to 50% of its original area. It is undesirable for the value to be lower than the protocol value.

TABLE 7. CONTRACTION RESULTS OF VAPOR CROSSLINKED ARTIFICIAL SKIN USED IN ANIMAL STUDIES

Study 1.	VXL	Control
M_c	25,000±2,400	24,000±3,600
D_{50}	12.3±1.3	16.7±2.1
No. of animals	4	3

Study 2.	VXL	Control
M_c	N/A	9,300±1,300
D_{50}	15.6±2.8	18.5±3.3
No. of animals	4	3

Table 7. shows that in both studies, the VXL membrane performed worse than the protocol membrane. While the two protocol values are not separated by more than one standard deviation, the two VXL values are. Granted, insufficient animals were run to state conclusively that the acetic acid vapor made a difference (especially given the high M_c values in the first study). However, this difference and the results of the rehydration study do indicate an avenue to be explored.

The two major differences between the solid state crosslinking procedure

investigated in this research and the aqueous crosslinking procedure are 1) the wet processed membranes are fully rehydrated in pH 3 acetic acid solution prior aldehyde treatment and 2) the concentration of the glutaraldehyde used in crosslinking. While glutaraldehyde concentration appears to have some effect on the density of crosslinking, the collagen present in the membrane is finely divided and thus should be independent of concentration effects.(12, 13)

The preliminary aqueous rehydration does not affect the eventual crosslink density. However, it does affect the collagen fibers in the membrane. That is, the fibers are fully swollen before they are crosslinked or 'fixed' in glutaraldehyde. This could be important on the cellular level as far as how the collagen/GAG is appreciated by the wound.

The animal trials should be done with two controls and four different types of vapor crosslinked material. In addition to a control produced by the wet crosslinking protocol, a wet crosslinked control should be produced with the acetic acid deleted from the rehydration and crosslinking steps.

The four VXL skins should be produced with the vapor exposure step either before or after the DHT step. As well, the VXL skins should be produced with or without a thirty minute exposure to just water and acetic acid vapor before the introduction of the glutaraldehyde vapor.

The use of a control that was rehydrated without acetic acid would provide insight from a different point of view. The only difference it would make is in the degree of swelling of the collagen prior to crosslinking.

The matrix of VXL membranes would determine if the rehydration step was important to the performance of the artificial skin. It has been demostrated that the exposure to water and acetic acid vapor prior to crosslinking does not affect the equilibrium M_c value.

APPENDIX I

Glutaraldehyde is a water soluble, nonflammable, active aldehyde of nominal composition: OHC $(CH_2)_3$ CHO. It has a specific gravity of 0.72; a freezing point of (-14)° C.; and a vapor pressure of 17mm Hg at 20° C. When heated, it decomposes at 188° C.(23)

It is most often supplied in 75%, 50%, and 25% w/w, aqueous solution. In the lowest concentration, it can still cause severe chemical burns and should be handled with eye and skin protection under adequate ventilation.(24) In this study, all actions involving glutaraldehyde were carried out with the appropriate safety equipment.

A volume containing part aqueous solution and part vapor (air, water vapor, and aldehyde vapor) has a mole fraction of 4% aldehyde and 96% water for every mole of solution vapor at 20° C., or a mole fraction of 0.8% aldehyde in the overall vapor mixture.

APPENDIX II

The data presented in Table A2.1 represent the average of two separate runs of one sample per type of collagen. The tests were performed in the MIT Fibers and Polymers Laboratory by Mr. Scott Berceli '85. The procedure is described in the text. As can be seen in the table, the lysine residues are the only amino acid to show a significant change due to glutaraldehyde exposure.

Table A2.1 RESULTS OF AMINO ACID ANALYSIS
(RESIDUES PER 1000 RESIDUES)

	Type of Collagen						
Amino Acid	A	B	C	D	E	F	G
Hydroxyproline	91	90	90	85	105	90	98
Aspartic Acid	48	44	44	42	46	47	44
Threonine	17	17	19	18	19	19	15
Serine	34	32	35	33	33	35	32
Proline	125	123	132	129	123	121	124
Glutamic Acid	81	79	85	79	79	78	76
Glycine	324	324	324	324	324	324	324
Alanine	110	105	104	104	118	97	98
Methionine	9	6	9	7	9	9	9
Isoleucine	14	16	17	14	15	14	15
Leucine	28	29	31	29	29	27	30
Tyrosine	5	4	3	3	5	3	4
Phenylalanine	15	16	15	15	15	17	17
Lysine	31	28	19	5	5	4	5
Histidine	8	6	6	8	7	7	7
Arginine	74	63	66	68	63	67	66

A) native bovine collagen.
B) protocol collagen/GAG membrane after DHT step.
C) aqueously crosslinked protocol collagen/GAG membrane.
D) vapor crosslinked protocol membrane, 1 hour at 35° C.
E) vapor crosslinked protocol membrane, 1.5 hours at 35° C.
F) vapor crosslinked protocol membrane, 2 hours at 35° C.
G) vapor crosslinked protocol membrane, 3 hours at 35° C.

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