CHARACTERIZATION OF COLLAGEN-MUCOPOLYSACCHARIDE COMPOSITE MATERIALS BY ELECTROCHEMICAL TRANSDUCTION

by

Miles R. Palmer

Submitted in Partial Fulfillment

of the Requirements for the

Degree of Bachelor of Science

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Abstract

Diffusion potentials are measured across collagen and collagen-mucopolysaccharide materials. The materials are similar in composition to those being studied as potential skin graft materials by the fiber and polymers research group in the department of mechanical engineering. The volume charge as a function of pH is determined by a calculation employing the Teorell-Meyer-Sievers theory of diffusion through charged membranes. The volume charge is related to the physical and chemical properties of collagen and mucopolysaccharide through examination of the structure and charging behavior of the two materials. The volume charge is correlated with crosslink density and with MPS content in the composite materials. The effects of varying pH during crosslinking upon the charging behavior is examined. The hysteresis effect exhibited by collagen is examined in both pure collagen and composite materials and found to occur irreversibly when certain bounds of pH are exceeded. It is hypothesized that the hysteresis effect is due to conformational changes in collagen due to greater interfiber electrostatic repulsion at high and low pH. It is concluded that the experimental technique employed is a very useful one by which to study polyelectrolytes in the solid state. It is suggested that the technique provides a very sensitive indicator of conformational changes or chemical reactions involving ionizable groups.

Thesis Supervisor: Alan J. Grodzinsky
Title: Assistant Professor of Electrical and Bioengineering

Acknowledgements

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I would like to thank Mike Picheny for his help in the computations involved in this work and for the work he did in building the apparatus and writing the background for his own thesis, which helped immensely in my own work.

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CHAPTER 1

INTRODUCTION

For some time a research group in the M.I.T. mechanical engineering department's fiber and polymers laboratory have been seeking to design a material which could serve as temporary grafts on burns in human patients. The group, headed by Professor I.V.Yannas, has chosen a composite material made up of collagen and a mucopolysaccharide for their present studies. The choice of collagen and mucopolysaccharides was motivated primarily by two facts. Collagen is the major structural protein in skin and various mucopolysaccharides are major components of the so-called "ground substance" usually found associated with the collagen fibers in skin.

A study performed by a member of that group (Huang, 1974) analyzed the in vitro physical and chemical properties of collagen-mucopolysaccharide(MPS) membranes of varying composition. Implantation of the membranes in guinea pigs was also carried out to study their behavior in vivo. It appears that some of the physical and chemical properties of such membranes may be related to the effective volume charge present on the component molecules and fibers.

The topic of this thesis is the study of volume charge present in membranes similar to the ones being studied as potential skin replacement materials. The volume charge is

calculated from diffusion potentials measured across various membranes. The diffusion potentials were created by the imposition of a sodium chloride concentration gradient across the membranes. The model assumed in the calculations is the Teorell-Meyer-Sievers (Teorell, 1953) theory of diffusion through charged membranes.

The volume charge was studied for its usefulness in relating the physical and chemical properties of various membranes. It was found that a number of valuable inferences could be drawn from the experimental data and that the technique seems to be a very useful one for the study of polyelectrolyte materials in the solid state.

The crosslink density in a series of membranes was found to be directly related to charge density and calculation of crosslink density from the charge data was found to give good agreement with an established value. There was some indication that in vivo behavior of composite implants correlates well with charge character. The effect of MPS on the charge characteristics of collagen was clearly evident, and the effective charge density appeared to better indicate in vivo behavior than established measurements of MPS content was able to in at least one case.

A relatively large hysteresis effect was discovered in the charging behavior of collagen and collagen-MPS composites. It was hypothesised that the effect was due

to subtle conformational changes in the collagen which were induced by electrostatic interfiber repulsion at high or at low pH. The effect seemed to begin to occur at about pH 5 and at pH 9. If the hypothesis is correct, the technique could prove to be a very powerful one for the study of conformational changes in biological molecules and for the study of biochemical reactions involving ionizable groups.

In the second chapter the properties of collagen and of mucopolysaccharides are discussed. In the third chapter the experimental technique and apparatus are presented, along with the TMS theory. In the fourth chapter, the experimental results are given and discussed.

CHAPTER 2

THE STRUCTURE AND PROPERTIES OF COLLAGEN AND MPS AND OF A COMPOSITE MATERIAL

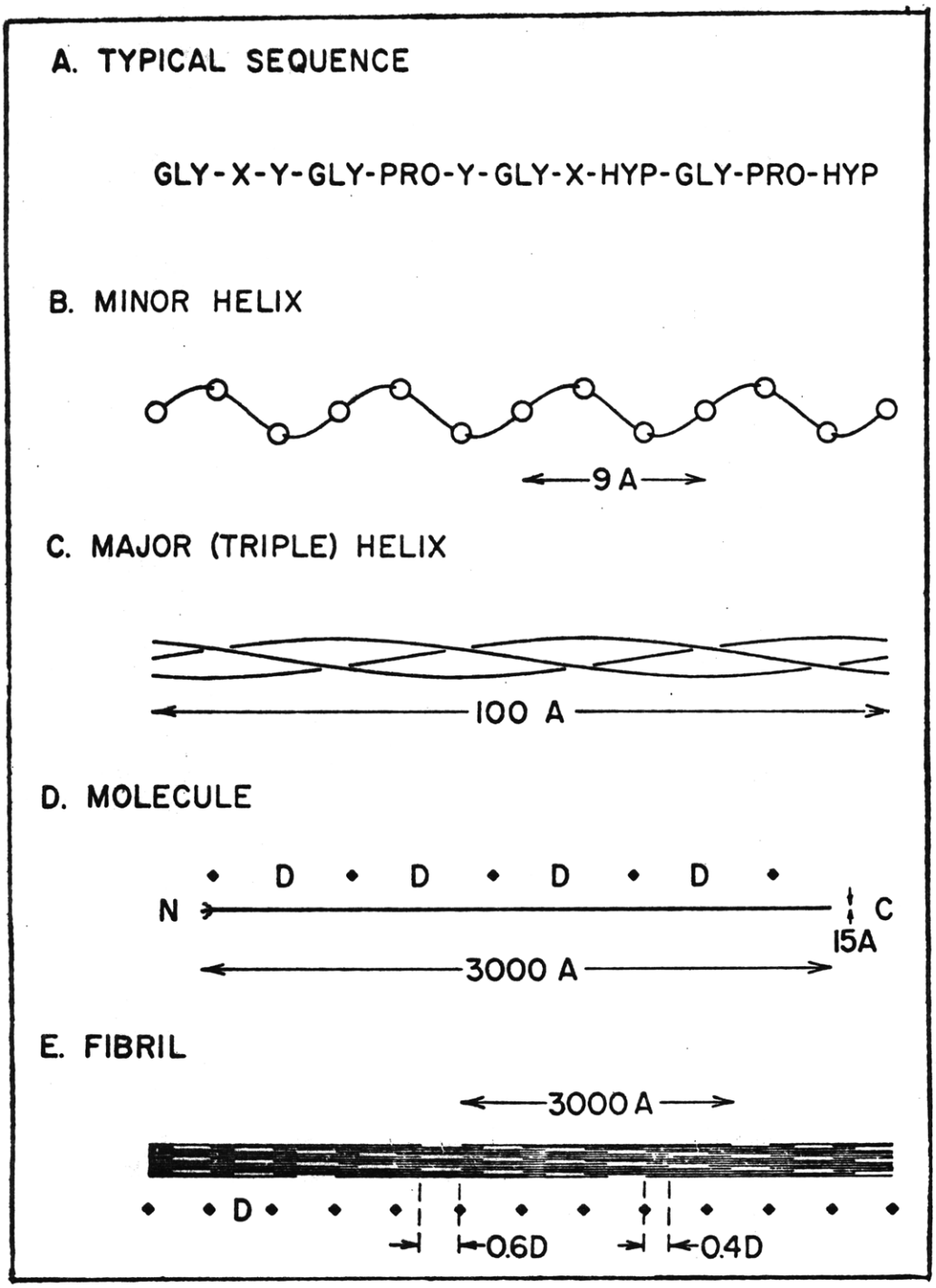
2.1 Collagen and MPS Structural Characteristics

Collagen is one of the most abundant naturally occuring proteins. Forming approximately 70% of the dry weight of human skin, it also makes up about one-third of the entire body protein. In the naturally occuring fibrous state it occurs in a complex microscopic configuration not yet fully characterized.

On the molecular level collagen is relatively well understood. Table 1 (Eastoe, 1967) shows the amino acid composition of human tendon collagen. The precise values vary somewhat from animal to animal or from tissue to tissue, but most varieties have roughly the same composition.

The amino acids are linked together to form polypeptide chains approximately 1000 amino acids in length with the repeating sequence as shown in (A) of Figure 1 (Piez,1969). Each chain has as its natural conformation a left handed helix as shown in (B) Figure 1. Three such chains wound together to form a right hand superhelix make up the collagen molecule, also termed tropocollagen and shown in (D) Figure 1.

The three chains are not usually identical. Most varieties of collagen have two identical chains complexed



Diagrammatic representation of several levels of order in collagen: A, primary structure; B, secondary structure; C and D, tertiary structure; E, quaternary structure. From Piez (1969).

Figure 1

AMINO ACID COMPOSITION OF COLLAGEN FROM

HUMAN TENDON (From EASTOE, 1967)

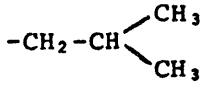
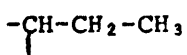
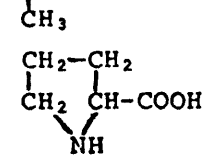
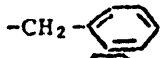
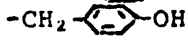
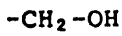
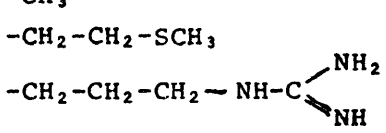
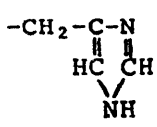
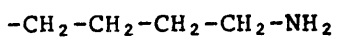
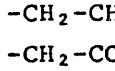
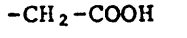
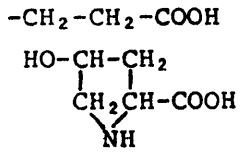
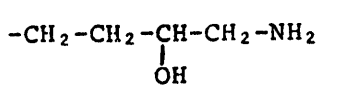
Trivial name	R in repeating sequence -NHCHRCO-; or imino acid formula	Number of amino acid residues per 1000 total residues
Alanine		110.7
Glycine		324
Valine		25.4
Leucine		26.0
Isoleucine		11.1
Proline		126.4
Phenylalanine		14.2
Tyrosine		3.6
Serine		36.9
Threonine		18.5
Methionine		5.7
Arginine		49.0
Histidine		5.4
Lysine		21.6
Aspartic Acid		48.4
Glutamic acid		72.3
Hydroxyproline		92.1
Hydroxylysine		8.9

Table 1

with a third chain which, among other things, has a higher histidine content (Piez, 1967).

The collagen molecules then associate into fibrils which have been detected by electron microscopy. The fibrils, as depicted in (E) Figure 1, are 500-1000 angstroms in diameter with an axial periodicity of 640 angstroms. Several structures have been proposed for the fibrils (Hodge and Petruska,1963;Veis,1967;Smith,1968). The structure proposed by Smith, shown in Figure 2, has been supported by X-ray diffraction studies(Miller and Wray,1971) and by molecular analysis (Segrest and Cunningham,1971). In any case the fibrils are stabilized by covalent intermolecular crosslinks.

In animal tissue collagen is usually found to exist as fibers with a diameter of about 2-10 microns (Yannas and Huang,1972). Since the aforementioned fibrils are only .05 to .1 microns in diameter, it is clear that many fibrils must somehow be bound together in each fiber.

A structure for the fibers and for the binding of the fibers in the tissue matrix has been proposed (Jackson and Bentley,1968). Their scheme, illustrated in Figure 3, involves interfibril binding by glycoprotein and interfiber binding by protein polysaccharide molecules. The glycoprotein molecules with their short carbohydrate side chains bind several fibrils together to form the 2-10 micron fibers

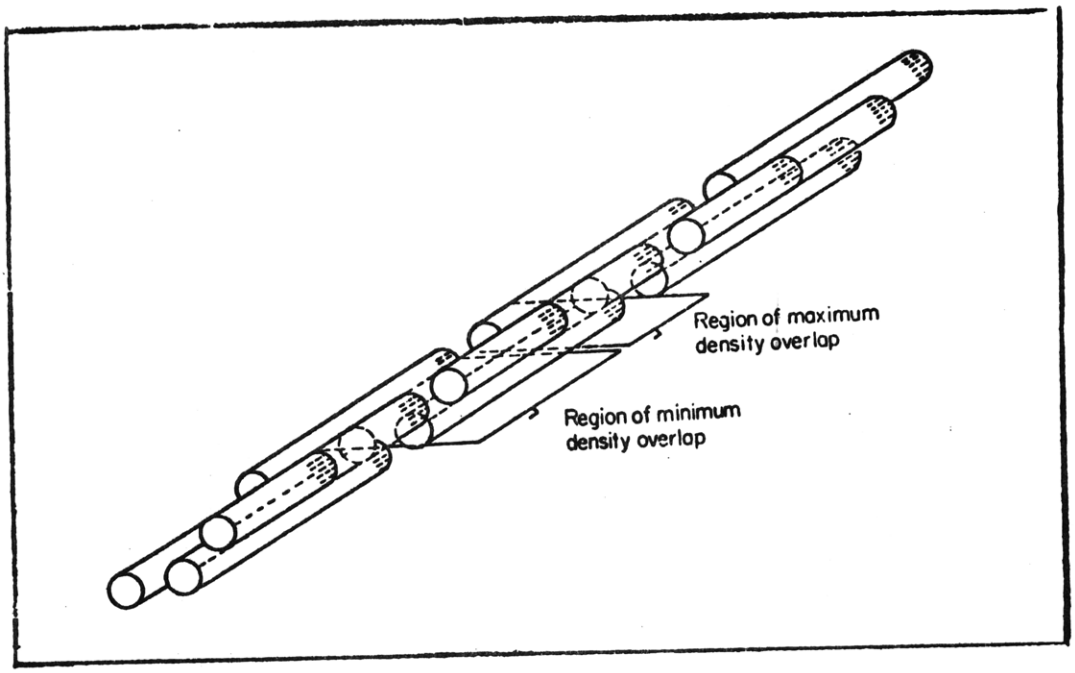


Illustration of proposed packing of tetramolecular units to form microfibrils. From Veis et al. (1970).

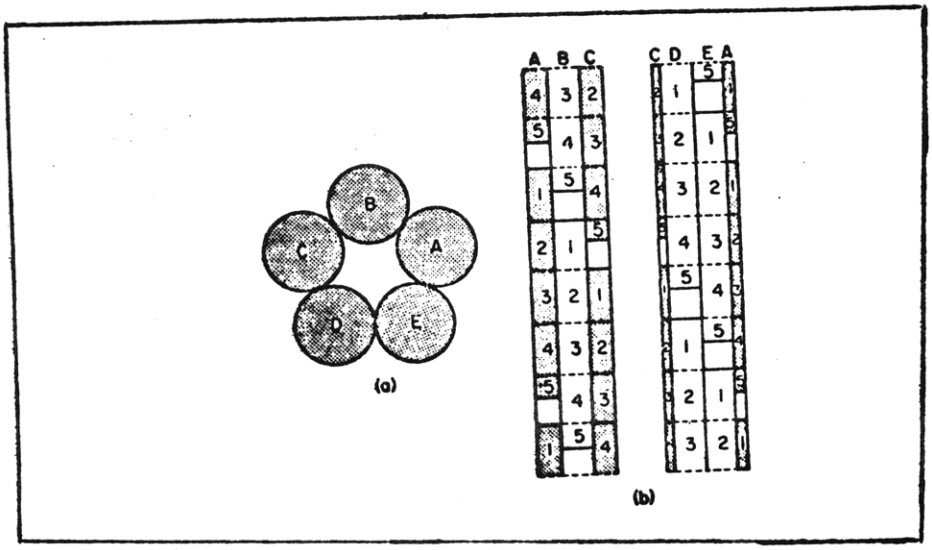
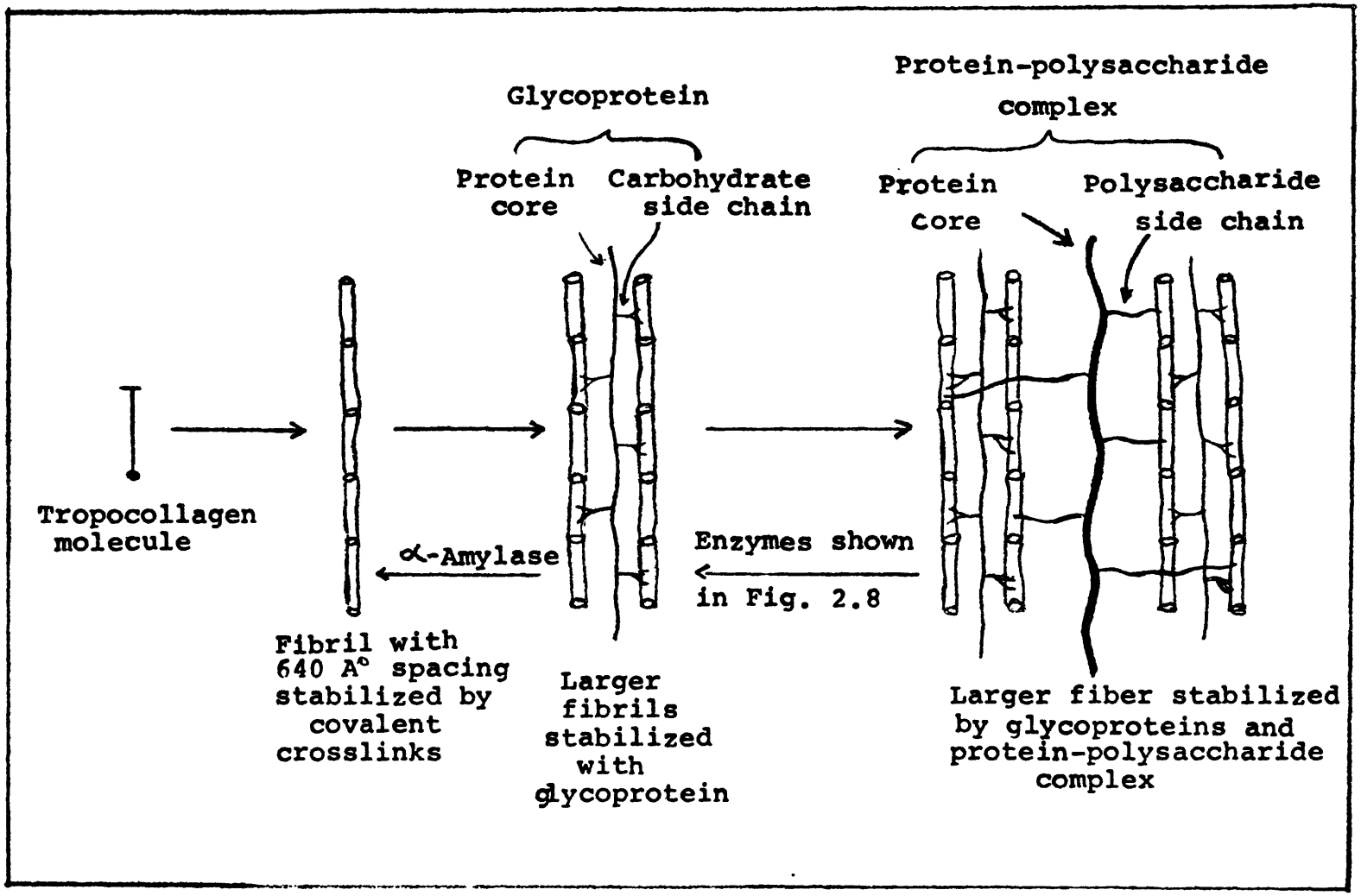


Illustration of proposed structure of filament composed of five chains of collagen molecules in regularly staggered array. The filament is shown viewed (a) in cross section and (b) perpendicular to its length from two opposite directions. From Smith (1968).

Figure 2



The role of glycoproteins and protein-polysaccharides in the stabilization collagen fibrils. From Jackson and Bentley (1968).

Figure 3

seen in tissue. The protein polysaccharide side chains, being much longer, bind the fibers together to form the widespread random matrix found in tissue. The protein polysaccharides then would form the majority of the "ground substance" found in tissue associated with collagen fibers.

The structure of a protein polysaccharide is shown in Figure 4. The carbohydrate side chains, without the protein core, are called mucopolysaccharides.

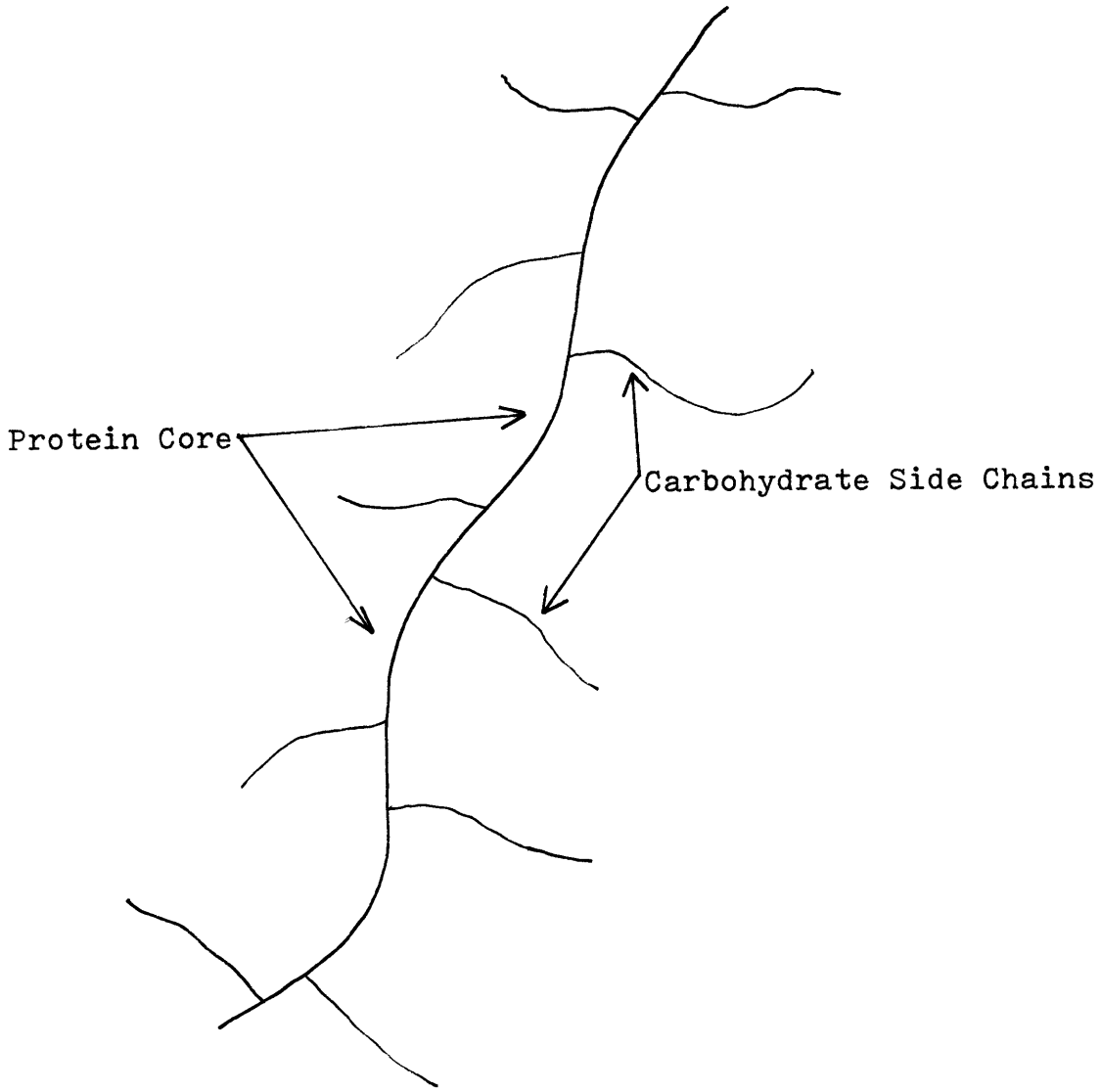
The structures of some typical mucopolysaccharides are shown in Figure 5. The mucopolysaccharide(MPS) with which this work is concerned is chondroitin-6-sulfate, which, with other mucopolysaccharides, is found in cartilage, bone, tendon, dermal skin, and sclera in mammals (Dodgson and Lloyd,1968). (See Figure 6.)

2.2 Charging Behavior of Collagen and MPS

Like most biological molecules both collagen and chondroitin-6-sulfate (C-6-S) contain many ionizable groups per molecule. The charging behavior of those groups influences the physical and chemical behavior of any material made up of collagen and/or C-6-S. Both collagen and C-6-S have acidic carboxyl groups which may deprotonate as follows.

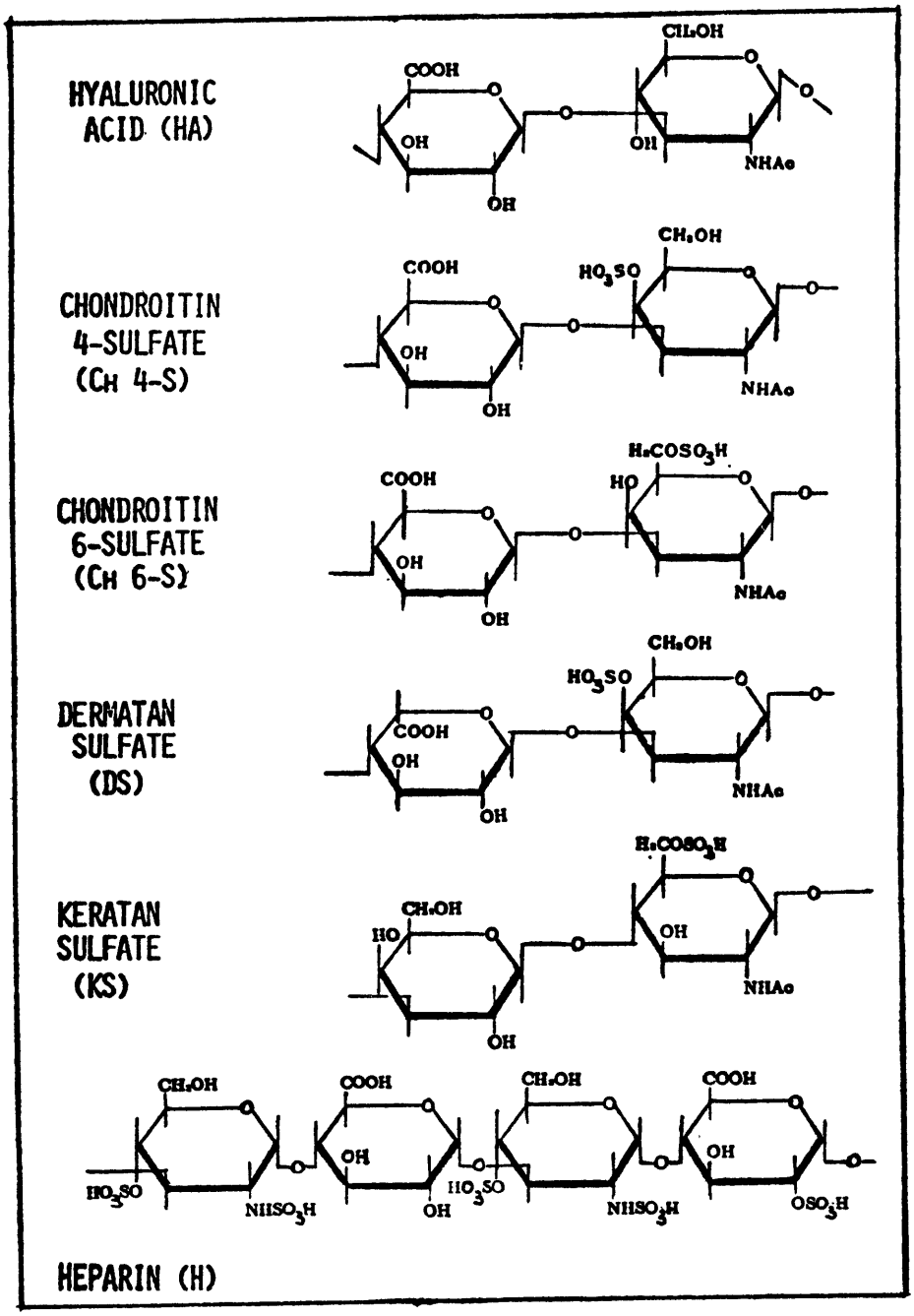
$$R\text{-}COOH = R\text{-}COO^- + H^+$$

The pKa of the carboxyl groups is defined as follows.



Structure of a typical protein polysaccharide.

Figure 4



Chemical structure of some acid mucopolysaccharides.
(Huang, 1974)

Figure 5

Figure 6

DISTRIBUTION OF ACID MUCOPOLYSACCHARIDES IN MAMMALIAN CONNECTIVE TISSUES

(From Dodgson and Lloyd, 1968)

Tissues	Polysaccharides							
	Hyaluronic acid	Chondroitin	Chondroitin 4-sulphate	Chondroitin 6-sulphate	Dermatan sulphate	Keratan sulphate	Heparin	Heparan sulphate
Umbilical cord	+	−	−	−	±	−	−	−
Synovial fluid	+	−	−	−	−	−	−	−
Cartilage	−	−	+	+	−	+	−	−
Bone	−	−	+	+	−	+	−	−
Tendon	+	−	−	+	+	−	−	−
Ligamentum nuchae	+	−	+	−	+	+	−	−
Skin (dermis)	+	−	±	±	+	−	+	+
Aorta	+	−	+	−	+	−	+	+
Vitreous humour	+	−	−	−	−	−	−	−
Cornea	−	+	+	−	−	+	−	−
Sclera	−	−	+	+	+	−	−	−

$$\log \frac{(R\text{-}COO^-)}{(R\text{-}COOH)} = pH - pKa$$

Thus as the pH increases the acidic caroxyl groups deprotonate. As the pH decreases they protonate. Equal concentrations of the protonated and the deprotonated species exist when the pH equals the pKa of the carboxyl group.

Chondroitin-6-sulfate also contains acidic sulfate groups. The sulfate functionality has a pKa of less than two, however. Therefore, in the pH range of interest, namely pH 3-11.5, the sulfate groups all remain ionized save for an insignificant fraction.

The amino acids lysine, hydroxylysine, and arginine have basic amino groups which may protonate as follows, thus lending positive charge to collagen.

$$R\text{-}NH_2 + H^+ = R\text{-}NH_3^+$$

The pKa is then defined as follows.

$$\log \frac{(R\text{-}NH_2)}{(R\text{-}NH_3^+)} = pH - pKa$$

Thus as the pH falls below the pKa, the amino groups protonate. As the pH goes above the pKa, the amino groups deprotonate.

Table 2 (Lehninger,1970) lists the pKa's of the amino acid side chain groups in collagen which charge in the range of interest. The pKa of the carboxyl group

Amino Acid	pKa of Side Chain Group
Aspartic Acid	3.86
Glutamic Acid	4.25
Histidine	6.0
Cysteine	8.33
Tyrosine	10.07
Lysine	10.53
Arginine	12.48

	pKa of Carboxyl Group
Chondroitin-6-Sulfate	3.4

Table 2

of chondroitin-4-sulfate is also given (Veis,1970). The pKa of the carboxyl group of C-6-S is assumed to be about the same, or about 3.4.

If the various groups in collagen ionized independently, then the charge present on collagen at any pH could easily be calculated from the data and formulas given. At pH3 for example the charge in moles per gram of collagen could be simply calculated by summing the numbers of positively charged groups from Table 1 and then dividing by the weight of all the amino acids together. The result is 0.72 mmoles per gram. At pH 11.5 the number of negatively charged carboxyl groups would be divided by the total weight to yield a charge of -0.68 mmoles per gram.

Similarly, since C-6-S has one sulfate and one carboxyl group per repeating subunit of 442 gram molecular weight, the charge at pH 3 would be -2.26 mmoles per gram, while it would be -4.52 mmoles per gram at pH 5 or higher. Chondroitin-6-sulfate is thus a much more highly charged molecule than collagen.

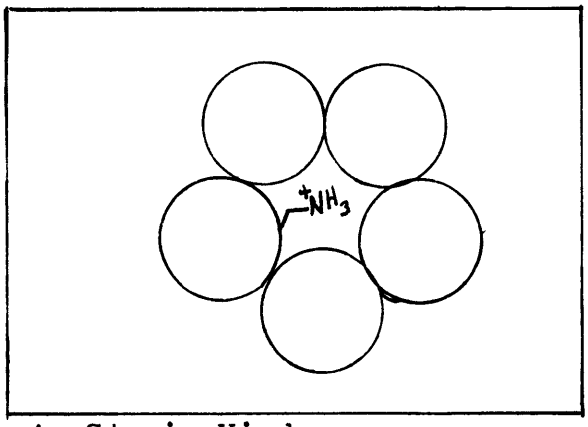
Collagen in the aggregate state, as detailed before, does not exist as a collection of independent amino acid chains. Mostly, it is present in the form of fibrils or fibers bound together in a matrix. It is obvious therefore that the calculations introduced serve only as a rough first approximation of the true charge. It might be imagined for example that only the charged groups lying on the outer

perimeter of a fibril would be accessible to the bathing solution for charging. The other groups would be blocked from charging by one of the interactions shown in Figure 7.

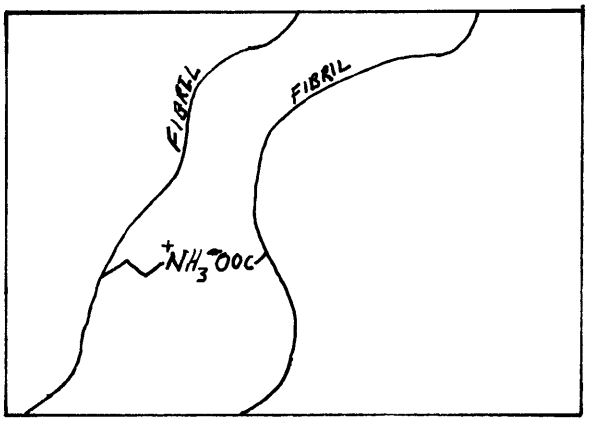
A group immersed in the inner portion of a fibril might well be stericly hindered as shown in (A). The group might be ionicly or covalently bound to another group as shown in (B) and (C). Hydrogen bonding or ionic shielding (D,E) may also occur. Any of the preceding interactions drasticly alters the charging behavior of the groups involved.

To determine the actual charging behavior of collagen in the aggregate state it is therefore necessary to perform measurements. The usual method of determining charge is by titration with acid or base. In Figure 8 is reproduced such a titration curve for collagen (Bowes,1948). Cowhide was very finely ground up by a mechanical means and dispersed in solution to produce the curve.

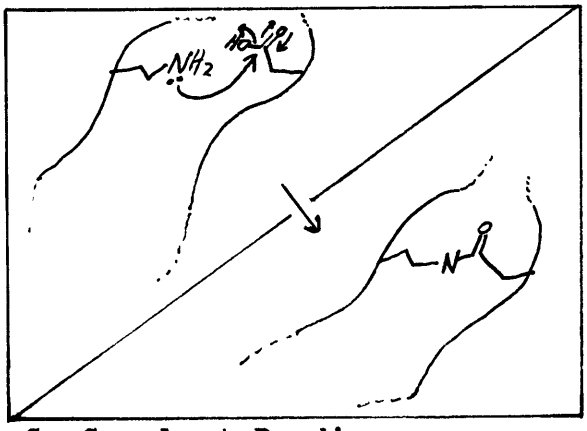
The charge present on the collagen fibrils can strongly affect the physical and chemical properties of a material made up of them. Consider the effect of varying pH on a collagen membrane made up of a mat of collagen fibers. At the isoelectric point all of the fibers would be effectively uncharged. Let that be the reference state. As the pH is decreased, the fibers become more and more positively charged. The fibers repel each other more and more and tend to move further apart. The membrane swells and more water



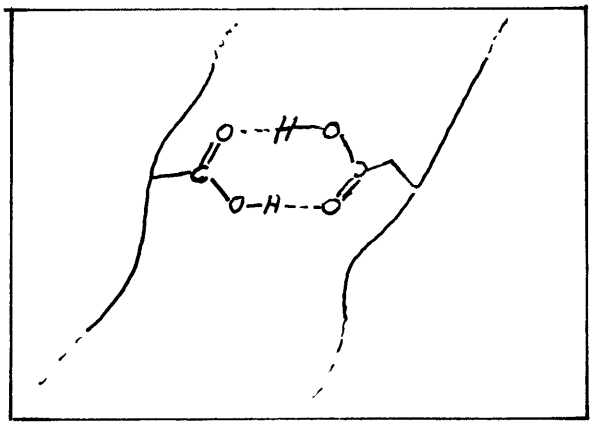
A. Steric Hindrance



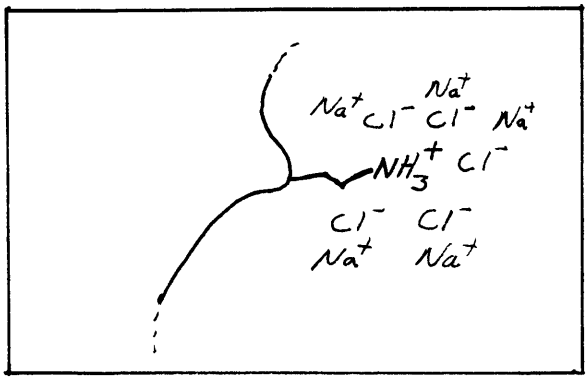
B. Ionic Binding



C. Covalent Bonding

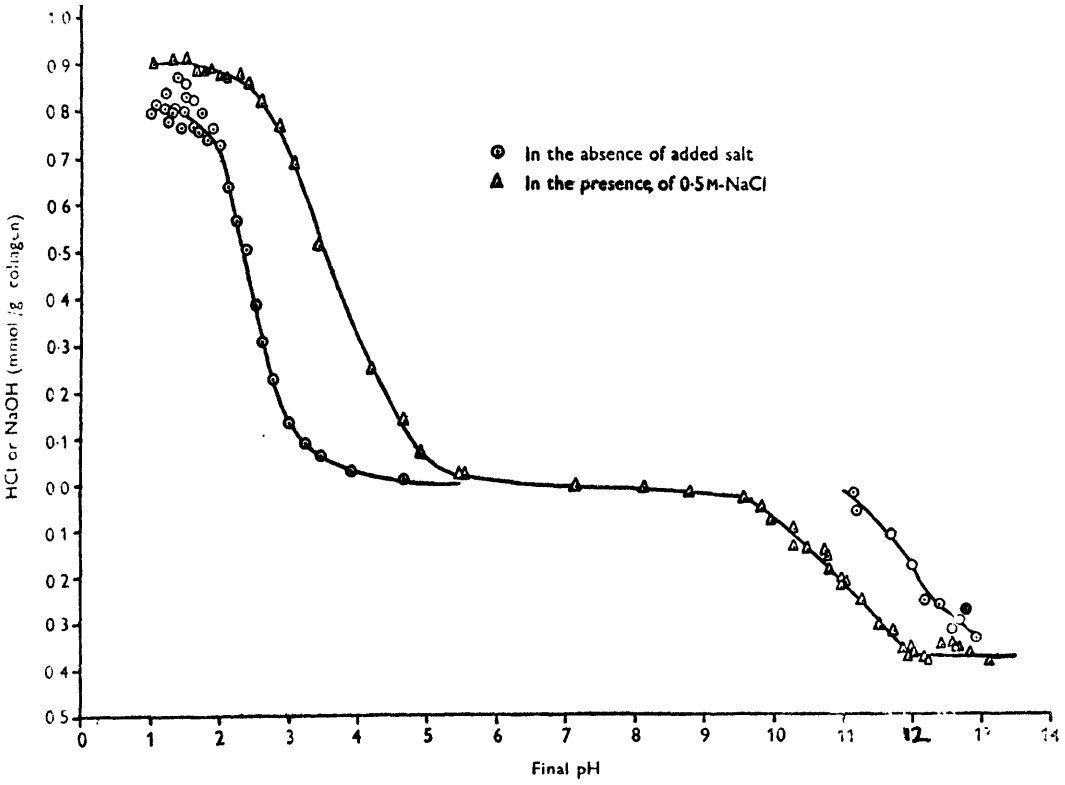


D. Hydrogen Bonding



E. Debye Shielding

Figure 7



Titration curves of collagen with and without sodium chloride.

Figure 8

is absorbed into the matrix. An analogous process occurs upon raising the pH. That phenomena is illustrated graphically in Figure 9 (Grodzinsky, 1976). As can be seen, if the ionic strength of the bathing solution is raised, Debye shielding decreases the interfibril repulsion, and thus the swelling ratio.

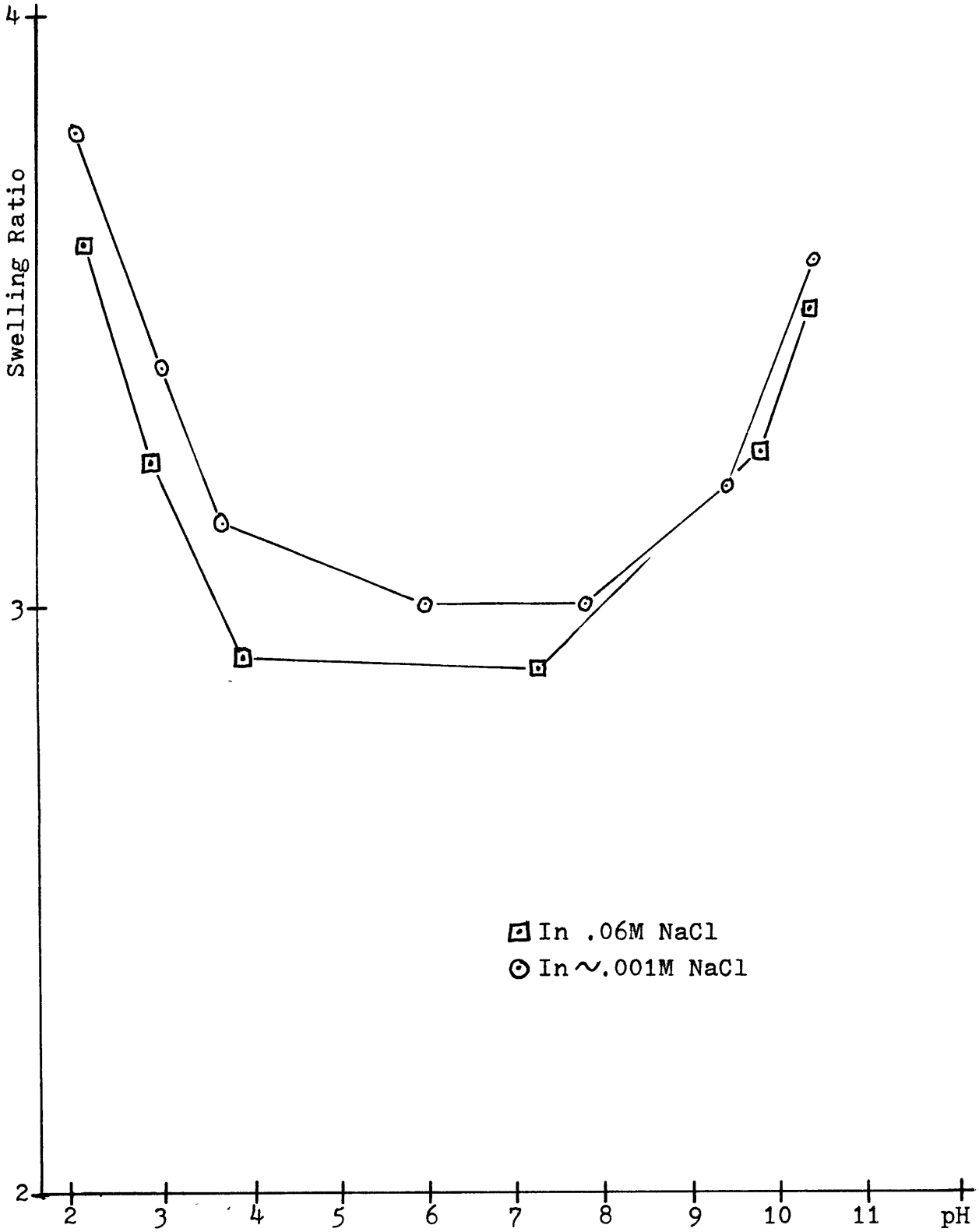
Another effect of swelling is to decrease the mechanical strength of the membrane. The more the fibrils repel one another, the more strained is the interfibril binding. Thus the membrane is more susceptible to mechanical rupture.

Binding to other materials by the membrane would also be affected by the charge. Skin for example is highly negatively charged under physiological conditions. When used in a graft it can therefore adhere very well to the underlying tissue, which is predominantly positive (Yannas,'76).

Solubility is another characteristic affected by charge. Fibrils of collagen are much more soluble in acetic acid than in water because they are more highly charged in acetic acid and thus bind more strongly to the polar solvent molecules.

2.3 Properties of Collagen-MPS Composite Materials

As mentioned in the introduction, a composite material formed of collagen and chondroitin-6-sulfate was chosen as a potential skin replacement material because of the large proportion of collagen and mucopolysaccharide in



Swelling in Sausage Casing Collagen as a Function of pH

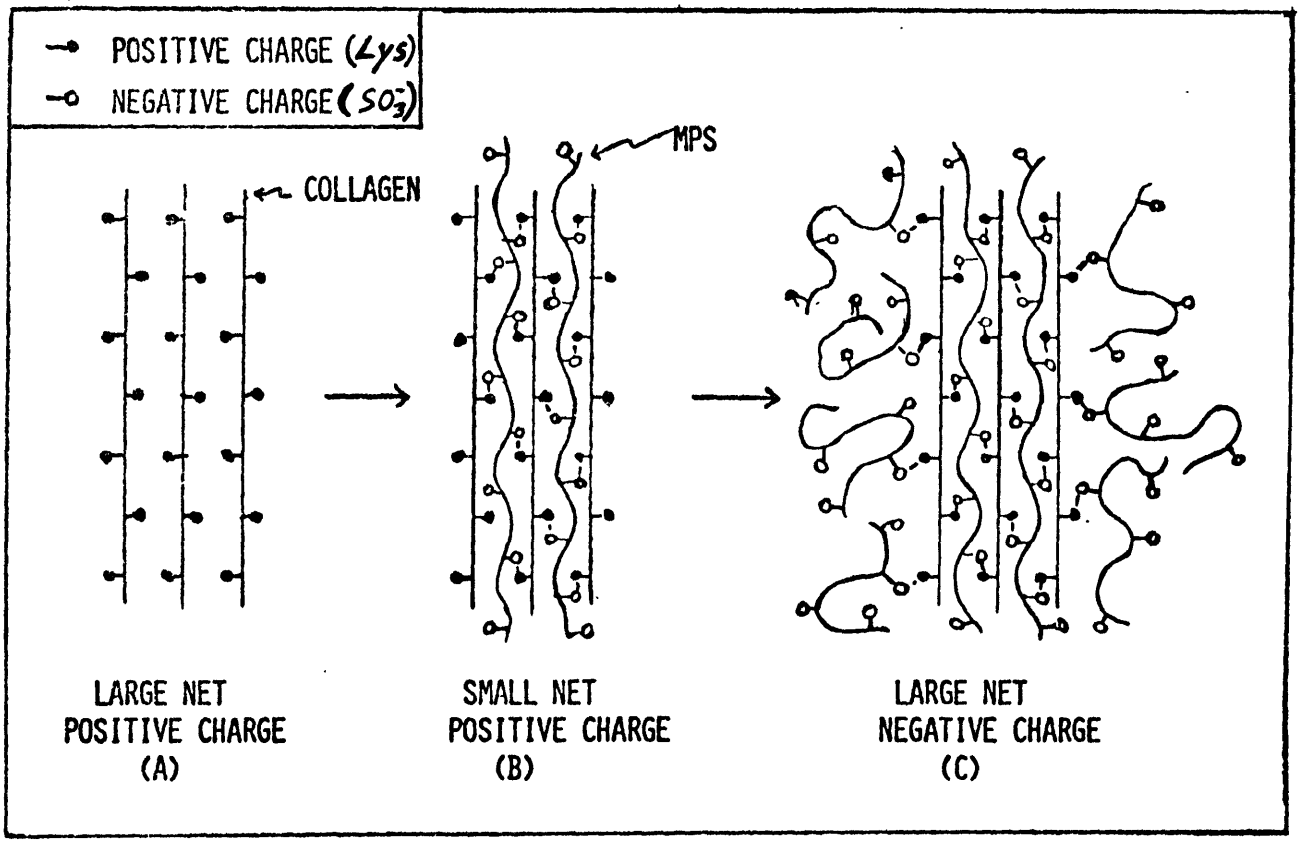
Figure 9

natural skin. The composite artificial material is formed by adding various amounts of C-6-S to a dispersion of collagen fibrils in a solution buffered at pH 3.2. Work by Podrazky and others (Podrazky, 1971) suggests that the amino groups of the side chains of arginine and lysine in collagen form an ionic complex with the sulfate groups of the MPS as shown in Figure 10.

At pH 3.2 collagen is positively charged. As more and more MPS is added, however, the net charge of the complex becomes less and less positive. The composite finally becomes neutral or even negatively charged. At the point when approximately 10% by weight of MPS has been added to the collagen, a fibrous precipitate forms (Gordon,1976). Precipitation probably occurs for two reasons. As the composite fibers become more neutral they repel one another less and are less interactive with the polar water molecules. Attractive intermolecular forces such as Van der Waals forces and hydrogen bonding then act to bind the fibers together.

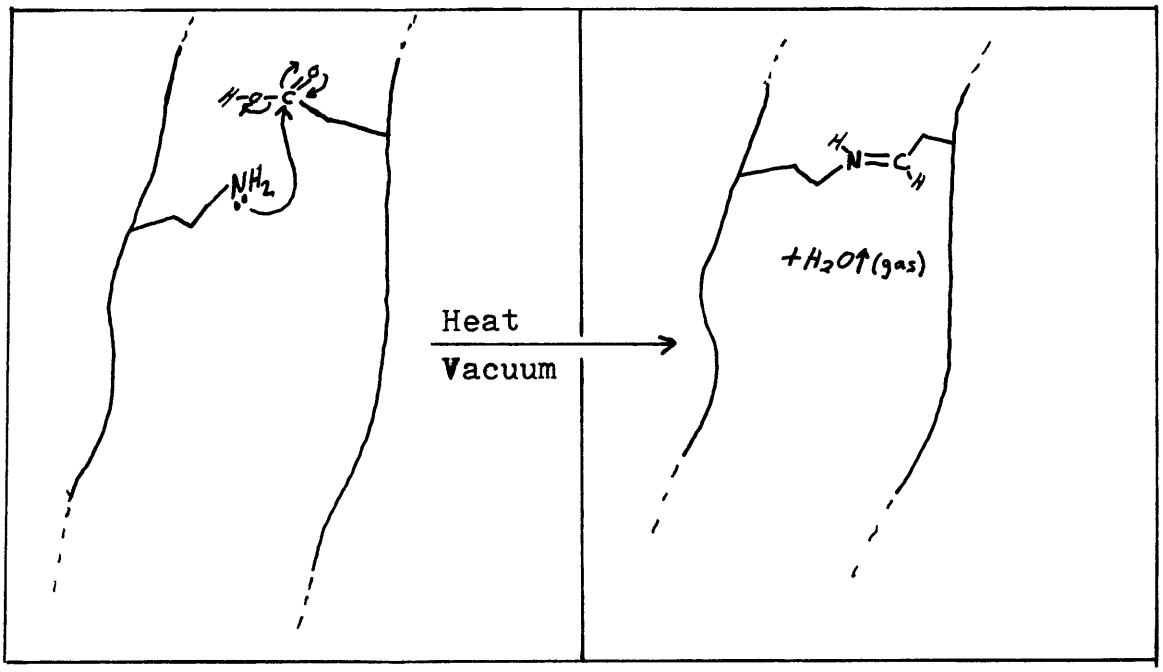
The precipitate is filter cast as a thin membrane, then crosslinked with glutaraldehyde. The crosslinking is believed to occur as shown in Figure 11. Since crosslinking eliminates charged groups, a heavily crosslinked membrane will differ in charging behavior from a lightly crosslinked one.

In Figure 12 (Huang, 1974) is a graphical representation of how physical behavior of the composite varies

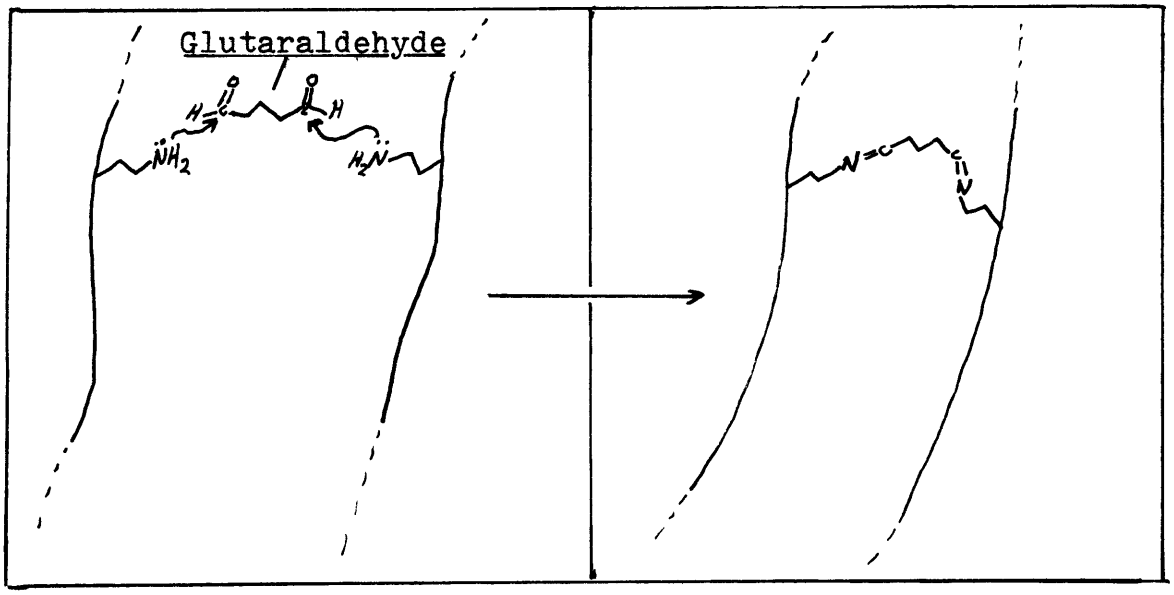


A model for explaining the variation in physicochemical properties of the collagen-Ch 6-S composite materials with the Ch 6-S content as shown in Fig. 12. See text for detailed description. (Huang, 1974)

Figure 10.

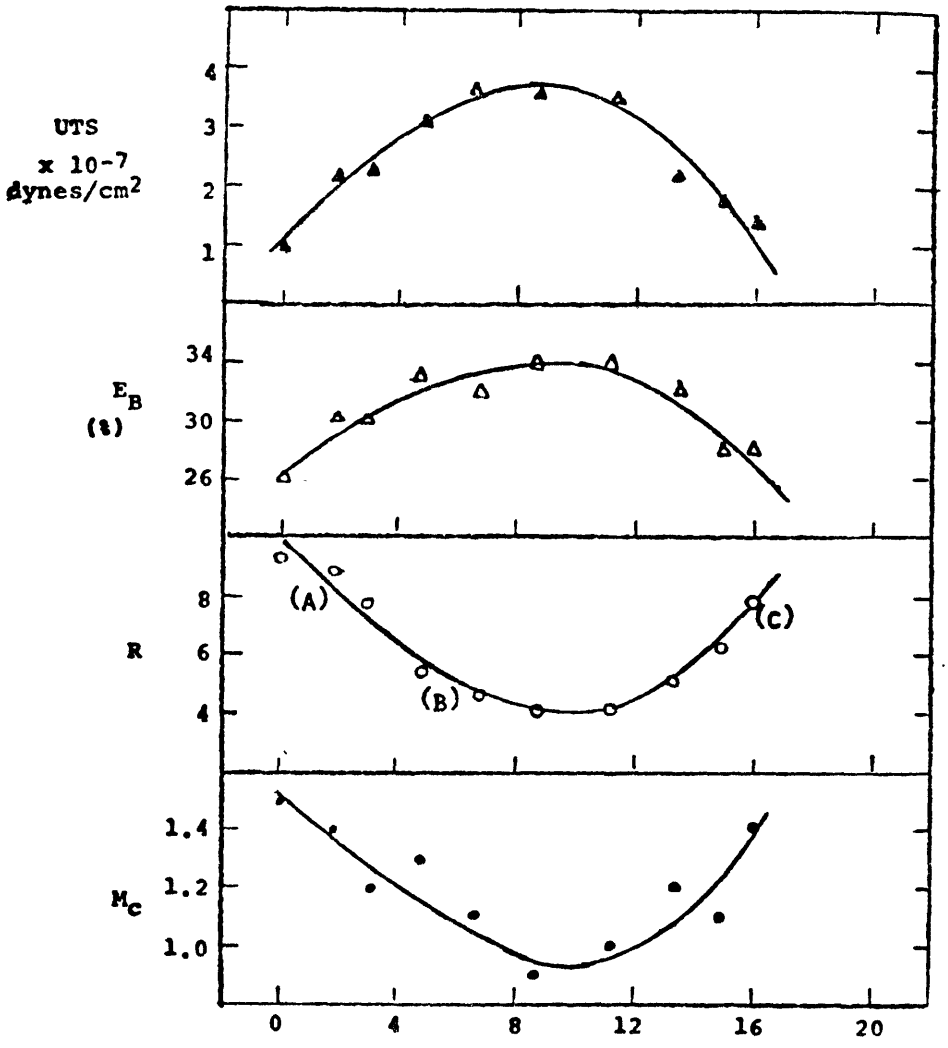


A. Dehydrative Crosslinking



B. Crosslinking with Glutaraldehyde

Figure 11



Comparison of a number of physicochemical properties of collagen-Ch 6-S composite materials containing varying amount of Ch 6-S. The properties compared are the ultimate tensile strength (UTS), elongation at break (E_B), swelling ratio (R) and number average chain molecular weight (M_c). (Huang, 1974)

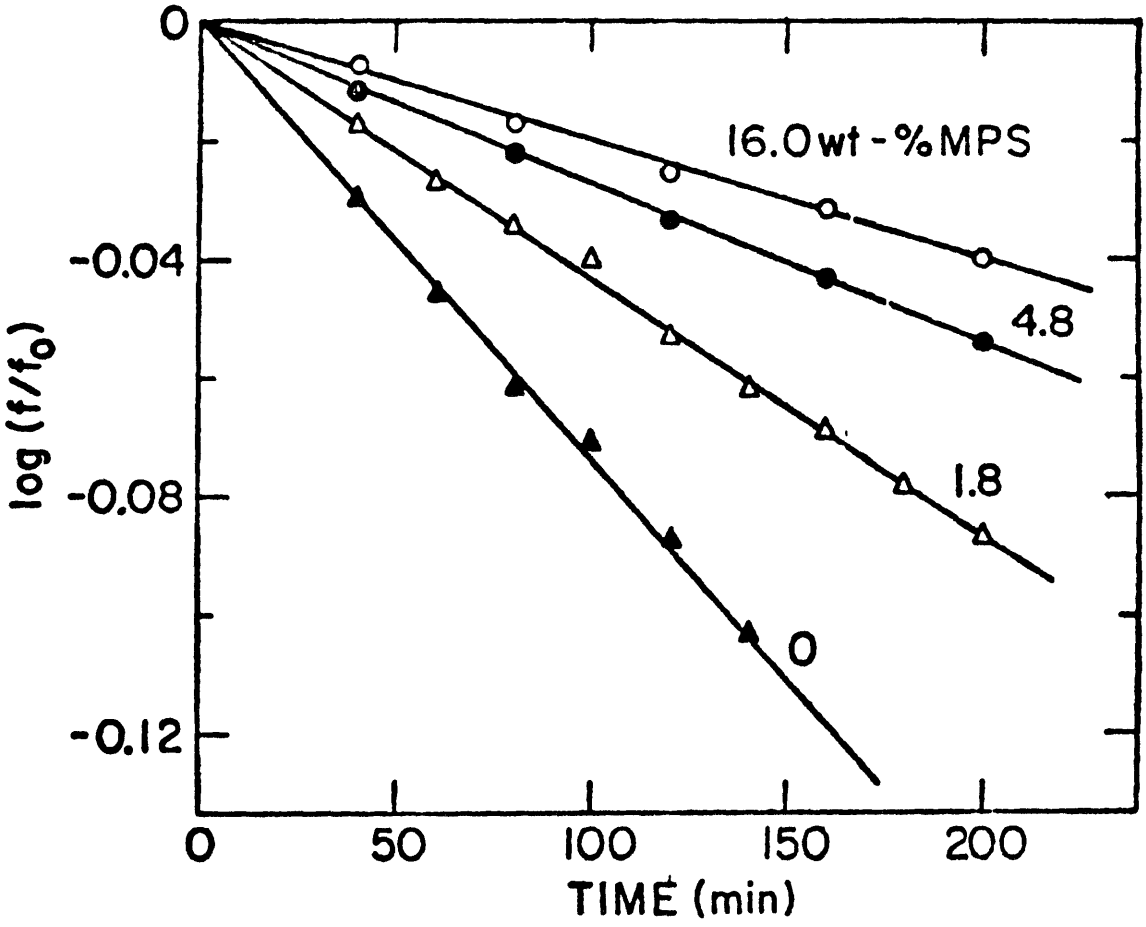
Figure 12

as a function of the amount of MPS. Looking at the varying net charge of the composite material is very informative. As described in the previous section, as the charge on each fiber in the composite varies, the interfiber repulsion, and thus the swelling, varies also. The mechanical strength is thus a function of the net charge also, but there is the added factor of a softening effect due to the MPS at high levels of MPS content (Milch,1966).

A most important property of the composite materials is their susceptibility to degradation by collagenase. Resistance to such degradation is essential if the composite material is to survive in vivo. A study by Yannas and others (Yannas, 1975) has analyzed the degradibility of composite materials as a function of MPS content. Their results are summarized in Figure 13. Since the amount of MPS present in a membrane may be inferred from the charge present on the membrane, the resistance to degradation may be indirectly inferred from the charge.

To be used as an artificial skin graft material, the composite membranes must be able to adhere well to the underlying dermis in wound areas. It appears from work done by Yannas and other that a highly negatively charged membrane will adhere best. (Yannas,1976)

Since so many physical and chemical properties depend upon or may be inferred from the charge present in the composite material, it is a very useful exercise to deter-



Force relaxation at constant extension of strips of reconstituted collagen conataining various amounts of chondroitin 6-sulfate, as indicated, and immersed in a collagenase solution. Fixed extension, 20 ± 2%; collagenase concentration, 40 units/cm^3; pH, 7.4; temperature, 37° C. (YANNAS, 1975)

Figure 13

mine that charge. As was mentioned earlier, standard chemical titration can give the charge per unit weight of titrated material. However, the effects which have been discussed are a function of charge per unit volume rather than of charge per unit weight. This fact becomes clear from the following argument.

The effects which have been discussed are directly the result of electrostatic forces present between charged components. Those forces are completely determined by specifying the charges present in the material, their position relative to one another, and the ionic atmosphere present between them. There is no dependence on the masses to which the charges are attached unless kinetic effects are important, which they are not here. Thus, since the volume charge density gives the charge in terms of relative position, while the charge per unit mass does not, the former is the much more useful quantity to determine. The volume charge density may of course be derived from the titration data provided the density of the material is known. The density varies, however. As does the swelling ratio, it depends upon the pH, the ionic strength, the crosslinking, and other factors.

A method which determined the volume charge directly would thus be much more useful than the standard technique of titration. This thesis is concerned with the application

of just such a technique to the study of the materials to be described, which are very similar to those being studied for their utility as skin replacements.

2.4 Preparation of the Membranes Analyzed

All of the membranes analyzed in this study were prepared in the fiber and polymers laboratory of the department of mechanical engineering. The preparatory procedures and compositions related here are those stated to me by Dr. Phil Gordon, a research associate in that lab.

The pure collagen membranes which are analyzed in this work were all prepared from collagen extracted from cowhide and supplied by the Department of Agriculture (Komanowsky, 1974). The collagen was freeze dried for storage. When needed it was dissolved in 0.5M acetic acid solution to form a dispersion of fibrils. Upon evaporation of the solvent in a petri dish, a cast collagen film remained. The membrane denoted M1 was removed and crosslinked in a 0.25% glutaraldehyde solution which was phosphate buffered at pH 7.4. Membranes 2A and 2B were crosslinked in the same manner except that the pH was 3.5. After 24 hours in the glutaraldehyde bath, all three membranes were washed in dilute dimedone solution to remove excess aldehyde. Membrane M7 was crosslinked by exposure to vacuum and a temperature of 104°C. for 24 hours.

Membranes M3,M4,and M5 were prepared by forming

a dispersion of collagen in phosphate buffer at pH 3.2 and then adding approximately 20% by weight of chondroitin-6-sulfate. The resulting fibrous precipitate was collected on filter paper by vacuum filtration. The membranes were then each crosslinked in a glutaraldehyde solution buffered at pH 7.4 for 24 hours. Membrane M3 was crosslinked in .00125% glutaraldehyde solution, M4 in .0025% solution, and M5 in .25% solution.

The membrane M6 was prepared by adding 10% by weight of C-6-S to a dispersion of collagen in acetic acid. Cast as a membrane by vacuum filtration, M6 was crosslinked in 0.25% glutaraldehyde solution buffered at pH 3.5 for 24 hours. All membranes were washed with dimedone and then deionized water.

Hexosamine assay (Swann, 1966) indicated the MPS content of M3,M4, and M5 to be 1-4% by weight, and that of M6 to be 5%. The hexosamine analysis was carried out prior to analysis of the membranes in this work.

CHAPTER 3

EXPERIMENTAL TECHNIQUE AND THEORY

3.1 The TMS Theory

The measurements actually made in this work were of the open circuit potential existing across the membranes studied. The potential resulted from the diffusion of NaCl due to the concentration gradient imposed across the membrane. The Teorell-Meyer-Sievers theory of diffusion through charged membranes may be used to calculate the volume charge present on a membrane for given activities and potential. (Teorell, 1953)

The theory models the potential drop across the membrane as the sum of a diffusion potential, ϕ_m, across the membrane, and Donnan potentials, $\phi_{D'}$ and $\phi_{D''}$ at the membrane liquid interfaces. The model is illustrated in Figure 14.

The potential to be measured across the membrane then is the following.

$$\phi = \phi_m + \phi_{D'} + \phi_{D''} \quad (1)$$

The Maxwell-Boltzmann distribution relates the ion concentrations inside the membrane to the concentrations in the bathing electrolyte as follows.

$$\bar{a}_{Na^+} = a_{Na^+} e^{+\phi_D F/RT}$$

$$\bar{a}_{Cl^-} = a_{Cl^-} e^{-\phi_D F/RT}$$

F=Faraday's Constant
a_i= activity of i
R= gas constant

Then $\bar{a}_i$ is the activity of i inside the membrane and at the interface, and a_i is the activity just outside the membrane. The activity is the concentration times an activity coefficient which will be determined from the Debye-Huckel equation for ionic activities (Huckel,1923). An activity is essentially an effective concentration.

Thus ϕ_0 becomes

$$\phi_{0'} = RT/F \ln \frac{\bar{a}'_{Na^+}}{a'_{Na^+}} = -\frac{RT}{F} \ln \frac{\bar{a}'_{Cl^-}}{a'_{Cl^-}}$$

Then it is apparent that

$$\frac{\bar{a}'_{Na^+}}{a'_{Na^+}} = \frac{a'_{Cl^-}}{\bar{a}'_{Cl^-}} = K_1 \quad (2)$$

Electroneutrality inside the membrane requires that

$$a'_{Na^+} = a'_{Cl^-} = a' \qquad \bar{a}_{Na^+} - \bar{a}_{Cl^-} + \bar{\rho} = 0$$

Here $\bar{\rho}$ is the volume charge present in the membrane. Solving then for K_1 gives

$$K_1 = \sqrt{1 + (\bar{\rho}/2a')^2} - \frac{\bar{\rho}}{2a'} \quad (3)$$

Similarly, K_2 is found to be

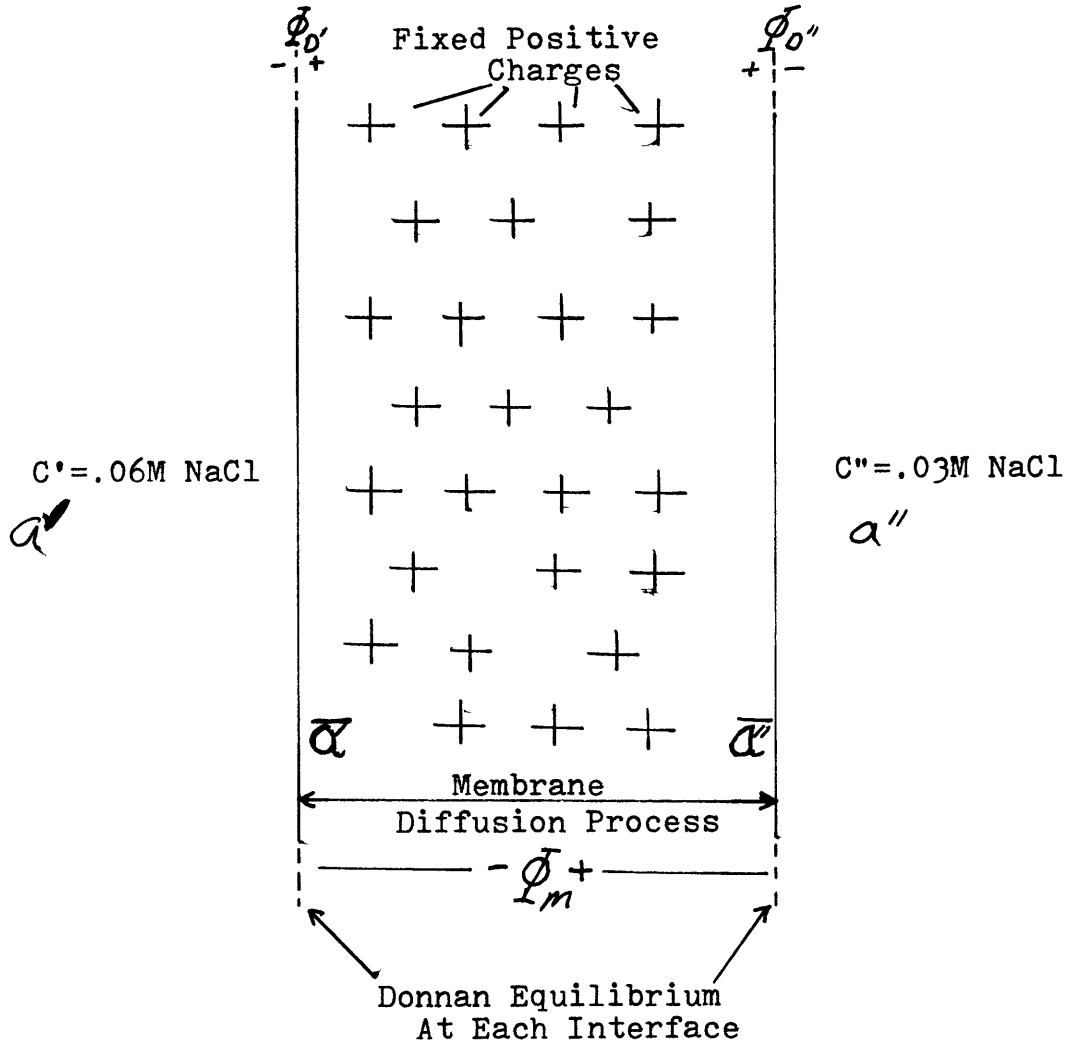
$$K_2 = \sqrt{1 + (\bar{\rho}/2a'')^2} - \bar{\rho}/2a'' \quad (4)$$

Therefore the sum of the Donnan potentials is

$$\phi_{0'} + \phi_{0''} = -\frac{RT}{F} \ln \frac{K_1}{K_2} \quad (5)$$

Thus given $\bar{\rho}$, a', and a", the internal activities, $\bar{a}'_{Na^+}$, $\bar{a}''_{Na^+}$, $\bar{a}'_{Cl^-}$, and $\bar{a}''_{Cl^-}$, can be caluculated from equations (2), (3), and (4). The Henderson equation, which follows, may then be used to calculate ϕ_m, where μ_i is the mobility of i.

$$\phi_m = \frac{RT}{F} \frac{\nu_{Na^+} - \nu_{Cl^-}}{\nu_{Na^+} + \nu_{Cl^-}} \ln \frac{\nu_{Na^+} \bar{a}''_{Na^+} + \nu_{Cl^-} \bar{a}''_{Cl^-}}{\nu_{Na^+} \bar{a}'_{Na^+} + \nu_{Cl^-} \bar{a}'_{Cl^-}} \quad (6)$$



Teorell-Meyer-Seivers Theory for Diffusion Through Charged Membranes.

Figure 14

The total membrane potential is then gotten from equation (1). The actual potential measured will not be ϕ, however. When electrodes are used in an electrolyte, there exists a liquid junction potential. In the case of most metals, that liquid junction potential is of much greater magnitude than ϕ. For that reason, silver chloride plated silver electrodes were prepared by a procedure described by Janz (Janz, 1961) and used in this study. The open circuit potential drop for silver chloride electrodes is well characterized and equals ϕ, where ϕ_e is given by

$$\phi_e = \frac{RT}{F} \ln \frac{a'_{Cl^-}}{a''_{Cl^-}} \quad (7)$$

The total measured potential then becomes

$$\phi = \phi_m + \phi_{D'} + \phi_{D''} + \phi_e \quad (8)$$

From equation 2-8, the following expression for ρ, the volume charge, may be derived.

$$\rho = a'' \sqrt{\frac{\left(e^{-2\phi_0 F/RT} - 1\right)^2}{4e^{-\phi_0 F/RT}\left(1 - \frac{a''}{a'} e^{-\phi_0 F/RT}\right)\left(e^{-\phi_0 F/RT} - \frac{a''}{a'}\right)}}$$

Several assumptions are implicit in the foregoing analysis. They are as follows.

1. Potential variations due to pressure work done by swelling of the membrane are negligible.
2. Water transport is negligible.

3. The mobilities of the ions remain constant throughout the system.
4. The concentrations of the ions on the outer side of the liquid membrane interface equal the ionic concentrations in the bulk of the electrolyte. That is, there are no stagnant layers of liquid at the surface of the membrane due to inadequate stirring.
5. The ionic strength and activities are assumed to remain constant over the course of the experiment. That is, the addition of acid or base and the diffusion of ions through the membrane do not perturb the system significantly.

3.2 The Experimental Technique and Apparatus

The apparatus and technique utilized in this work is essentially that developed by Grodzinsky and Picheney (Grodzinsky, 1976). Somewhat more sophisticated instrumentation streamlined the procedure and perhaps led to more accurate experimental data.

The main departure in this work from previous work done by them is that the analysis of composite collagen-MPS materials is performed and an investigation of hysteretic phenomena associated with the materials is made.

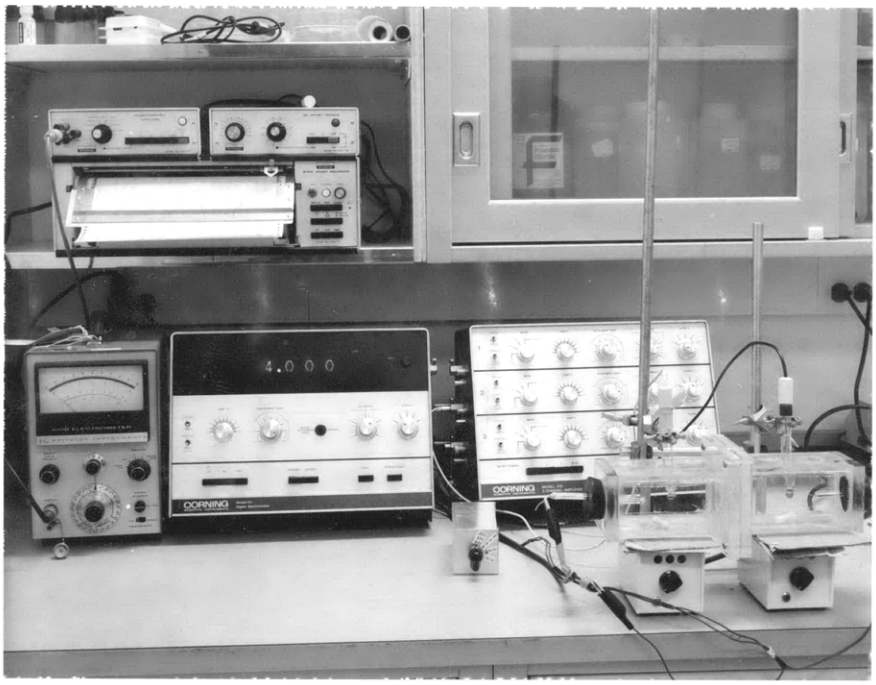
The technique employed in this work offers several advantages. Most importantly, it appears that the technique allows a great deal of information concerning the physical

behavior and the chemical makeup of a membrane to be determined. As will be discussed later, such things as crosslink density, effective charge density changes due to addition of MPS, and subtle conformational changes can be detected. A very practical advantage is that the method is capable of analyzing intact membranes in exactly the same form as would be used in actual implantation studies. Since the technique is nondestructive, analyzed samples could afterwards be used as implants.

The apparatus pictured in Figure 15 and diagrammed in Figure 16 was used in this work. The potential was monitered with a Keithly Model 610B electrometer, the output of which drove a Klumberger strip chart recorder. Chloride ion concentration and pH were measured using Orion and Corning electrodes. The pH electrodes were of the internal reference type. A Corning Model 101 digital displaying electrometer was used in conjunction with a Corning Model 103 three channel amplifier to read out pH and Cl^- concentration.

A four pole four position switch was used as shown in figure 16 to switch the reference or ground side of each electrode. It was found that simultaneous connection of two or more electrodes produced offset readings, apparently due to electrode-electrode potentials.

Silver chloride electrodes were prepared by the method



The Experimental Apparatus

Figure 15

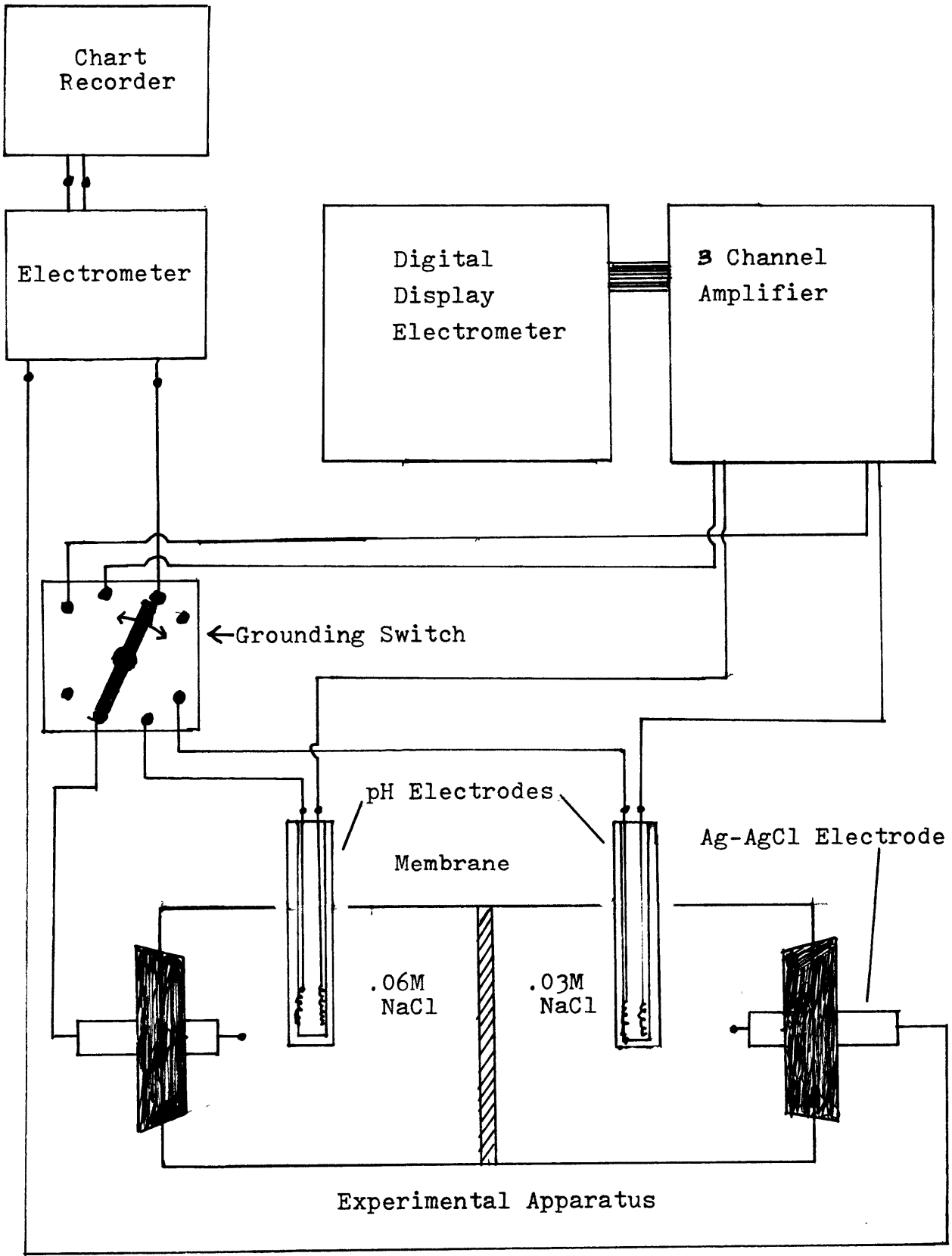


Chart Recorder
Electrometer
Digital Display Electrometer
3 Channel Amplifier
←Grounding Switch
pH Electrodes
Membrane
Ag-AgCl Electrode
.06M NaCl
.03M NaCl
Experimental Apparatus

Figure 16

outlined by Janz (Janz,1961) and used to measure potential.

Small pieces of corrugated cardboard were placed between the chambers on each side and the magnetic stirrers in order to insulate the apparatus from heat generated by the stirrers.

Volume charge was calculated from the measured potentials using an interative APL program written by Picheny (Picheny,1975) and reproduced in Figure 17.

3.3 Experimental Procedure

Several hours before each experiment, all equipment was turned on to allow time for warm up and stabilization.

All experiments were conducted at room temperature and atmospheric pressure.

Seven liters each of .06M and .03M NaCl were prepared from deionized water. These solutions were used in all experiments. Between experiments the silver chloride electrodes were kept in 1% NaCl solution and were short circuited. Before each experiment the electrode offset was measured and was always found to be less than .2mv. Between experiments, the membranes analyzed were kept in deionized water in a refrigerator to retard bacterial degradation.

All apparatus in contact with the salt baths was washed thoroughly with deionized water between experiments.

```
     V Z←PREDICT POT
[1]    'CONSTANT IONIC STRENGTH?'
[2]    →CHIS×ι'N'=(,⍞)[1]
[3]    'TYPE CONC FOLLOWED BY MOBILITIES'
[4]    CONC←⎕
[5]    MOBMAT←⍉((⍴POT),4)⍴CONC[1 2]∘.×CONC[3 4]
[6]    C1←(⍴,POT)⍴CONC[1]
[7]    C2←(⍴,POT)⍴CONC[2]
[8]    →COMPUTE
[9]   CHIS:→COMPUTE
[10]  COMPUTE:CONC←0
[11]   'NO. OF ITERATIONS?'
[12]   IT←⎕
[13]   TEMP←1↑DIFPOT←(⍴POT)⍴0
[14]  ITER:CHARGE←(POT-DIFPOT) WX C1,[0.5] C2
[15]   R1←((1+R1*2)*0.5)-R1←CHARGE÷2×C1
[16]   R2←((1+R2*2)*0.5)-R2←CHARGE÷2×C2
[17]   U1←MOBMAT[1;]×R1
[18]   U2←MOBMAT[3;]×R2
[19]   W1←MOBMAT[2;]÷R1
[20]   W2←MOBMAT[4;]÷R2
[21]   DIFPOT←-60×((U1-W1+U2-W2)÷U1+W1-U2+W2)×10⍟(U1+W1)÷U2+W2
[22]   →ITER×ι IT≥TEMP←TEMP+1
[23]   (60×10⍟R1÷R2),DIFPOT,((60×10⍟R1÷R2)+DIFPOT),POT,[1.5] CHARGE
     V
```

Figure 1

In each experiment one chamber was filled with .06M NaCl, the other with .03M. The membrane formed the wall separating the two chambers, being clamped taught between rubber O-rings.

The pH electrodes were calibrated using pH 4 and pH 10 buffer solutions. The chloride electrode was calibrated with standard 0.1M and 0.01M NaCl solutions.

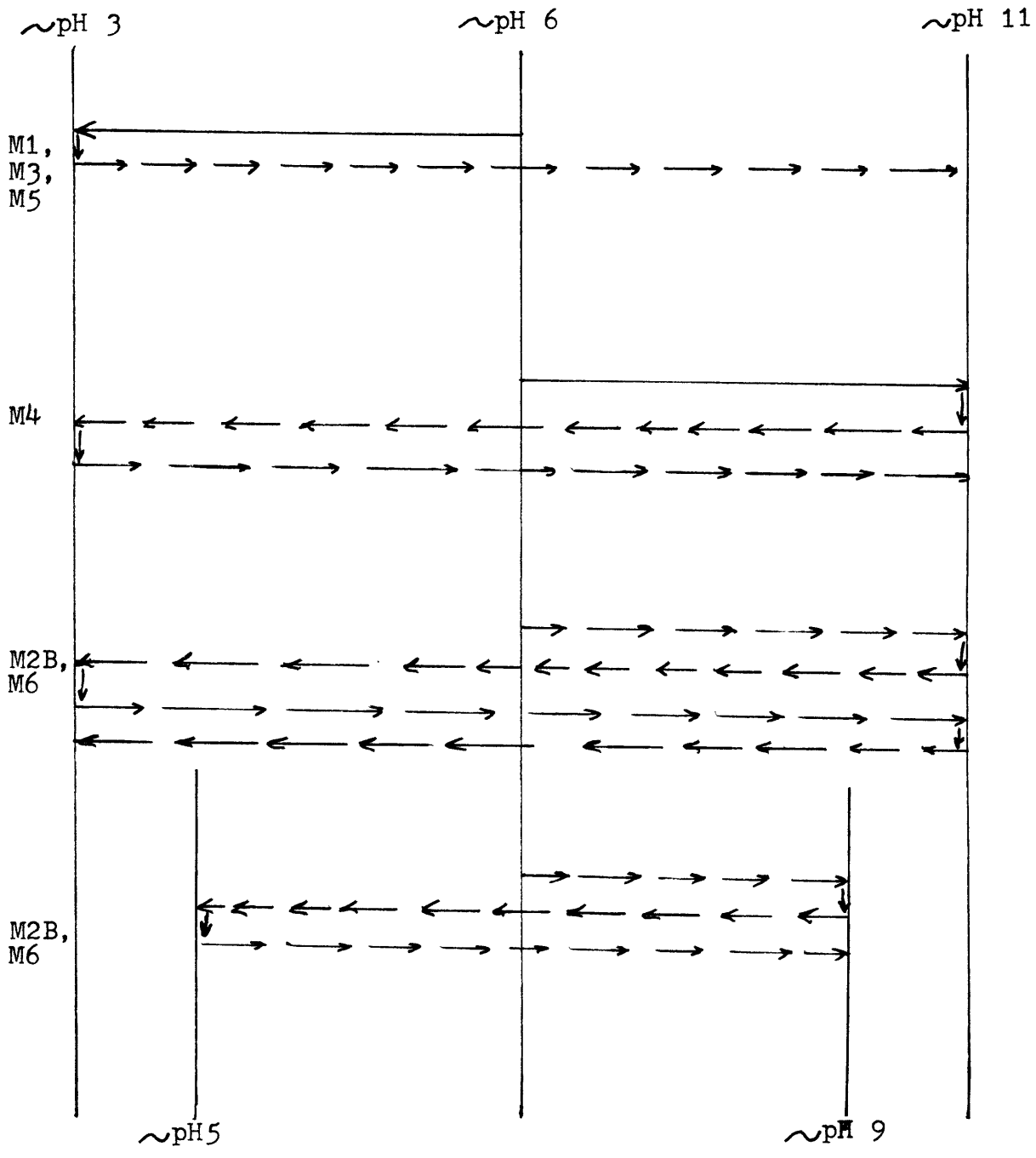
The stirrers were turned on and the initial pH and potential were measured. The side of lower salt concentration was taken as the positive reference. Acid or base was then added to reach the desired pH. The chart recorder was then observed to determine when the potential had reached an asymtotic value. After recording the asymtotic potential, the pH was again measured to ensure that it remained unchanged and equal on both sides of the membrane. The same sequence was repeated for each data point. The detailed outline of the sequence of pH changes made in the analysis of each membrane is summarized in Figure 18. The sequence and order of pH variation was found to influence the observed results, as will be discussed in the next chapter.

At some points in the longer experimental runs it was necessary to replace the salt solutions due to the larger amount of acid and base added. This was in order to minimize the perturbation of the salt concentrations.

At the end of each experiment the chloride ion

concentration was measured to determine if the salt concentrations on either side of the membrane had been perturbed. In all cases, less than a 10% change had occured

Several experiments were repeated days or weeks after the initial analysis. In all cases the experimental results proved to be repeatable to within .3-.4 millivolts.



Sequencing of pH Variation in the Experimental Analysis

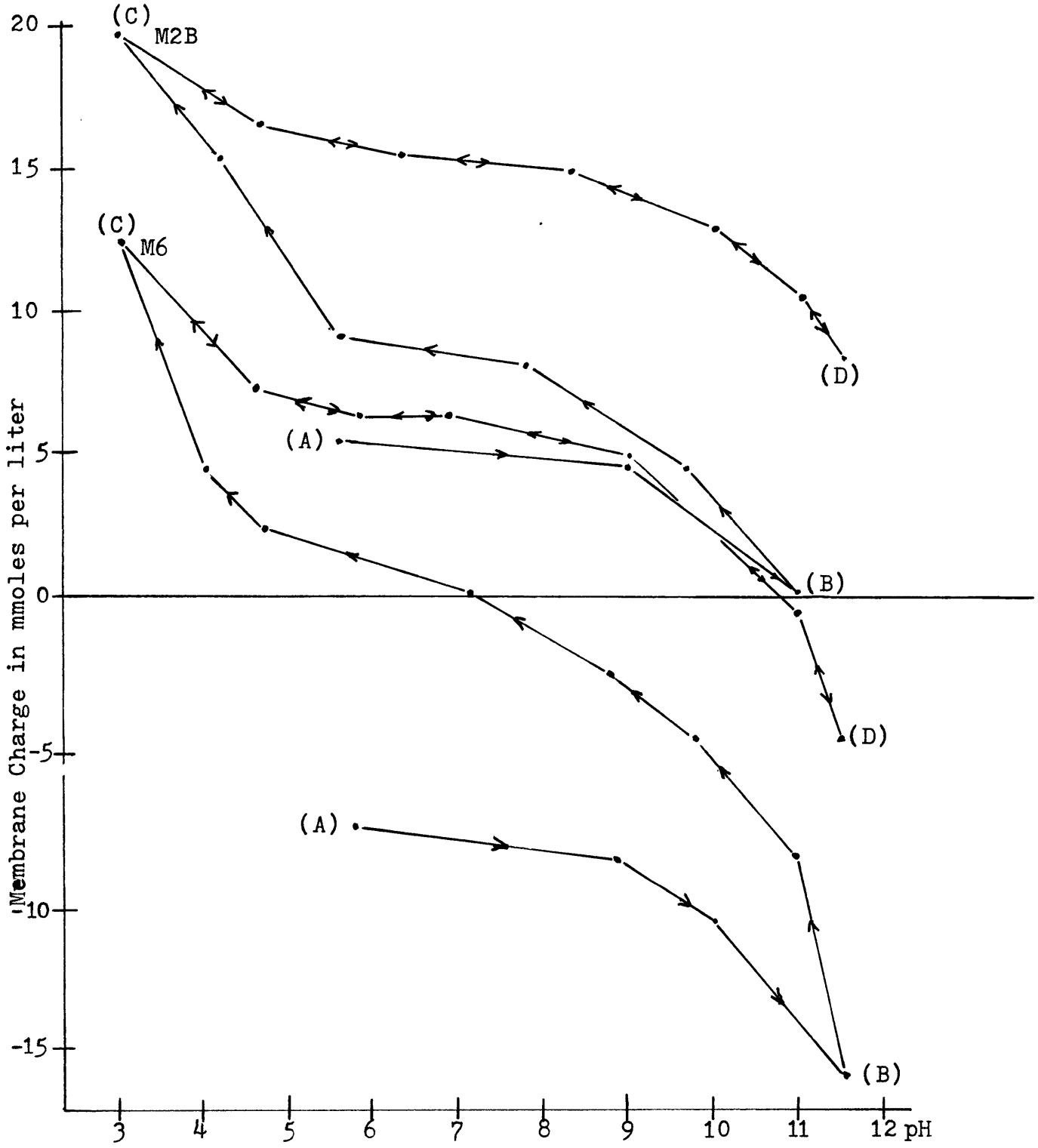
Figure 18

CHAPTER 4

DISCUSSION OF EXPERIMENTAL RESULTS

Figure 19 shows a plot of the volume charge present on membranes M2B and M6. M2B was pure collagen while M6 contained about 5% MPS. Beginning at the initial unperturbed pH of about 6 (A), base was added to reach point B (pH 11.5) The arrows indicate the direction in which the pH is varying over the curves. As can easily be seen, there is a large hysteretic effect. An important experimental fact, however, is that the hysteresis appears to be irreversible. That is, once pH 3 is reached, more hysteresis does not occur. Varying pH will produce charge on the membrane in accordance with the curve from C to D in both directions. The observed changes in net volume charge cannot be attributed to volume changes due to swelling, since in the case of M6, the net effect of the hysteresis has been to completely reverse the charge present on the membrane rather than simply change its magnitude. The hysteresis can only be due therefore to a net change in the effective number of ionizable groups in the membranes. Since essentially the same effect occurs whether or not MPS is present, the functional groups of collagen are apparently involved.

The net charge difference between branch AB and branch CD in both curves remains constant from pH 6 to



Membrane Charge of M6 (collagen-MPS composite) and M2B(pure collagen) as a Function of pH.

Figure 19

pH 11. This indicates that the groups affecting the hysteresis remained charged in that region. If they became neutral in that region, the effect would disappear.

The only groups which remain charged in that region are aspartic acid, glutamic acid, and arginine. Thus the effect might be due to effective elimination of basic carboxyl groups or the creation of new accessible basic arginine groups. Carboxyl groups might be eliminated by some sort of crosslinking reaction as shown in Figure 11. However, the reaction in that case would also eliminate basic amino groups. The net effect would therefore be zero in the region of pH in which both groups would have been charged prior to the crosslinking, namely pH 6 to pH 10.

Another possibility is that conformational changes occur in the membranes which act to stericly hinder or otherwise block the carboxyl groups as shown in Figure 7 (D,E). Since new basic groups could not have been introduced into the membrane, the creation of new accessible basic groups could only occur by conformational changes which make accessible arginine groups which were previously hindered.

In looking at the hysteresis gap between branch AB and branch BC, it is apparent that the gap is not as wide in the curve for M2B as for M6. The reason is that the AB branch terminates at pH 11.0 in the case of M2B and

at pH 11.5 in the case of M6. It was found that the pH could be varied approximately from pH 5 to pH 9 without significant hysteresis effects. That is, the initial pH of 5.8 could be perturbed by the addition of base to reach pH 9, and upon addition of acid to return to pH 5.8, the charge would return to its original value. Further excursion from neutral pH leads to hysteresis.

Such observations support the hypothesis that the hysteresis is due to conformational changes. Such changes in conformation should reasonably occur at high or low pH more readily than at neutral pH due to the charge repulsion effects between fibers which have already been mentioned. It is in fact well known that much more drastic conformational changes occur under extremely acidic or basic conditions. Collagen, it is known, even denatures under sufficiently severe conditions, its conformation changing from that described in Chapter 2 to a randomly coiled array of protein chains as observed in gellatin. (See Figure 20)

The driving force for the proposed conformational changes is electrostatic repulsion. Therefore the conformational changes should be influenced by varying the ionic strength. Such a study would be an interesting one.

It seems reasonable to conclude that the conformation

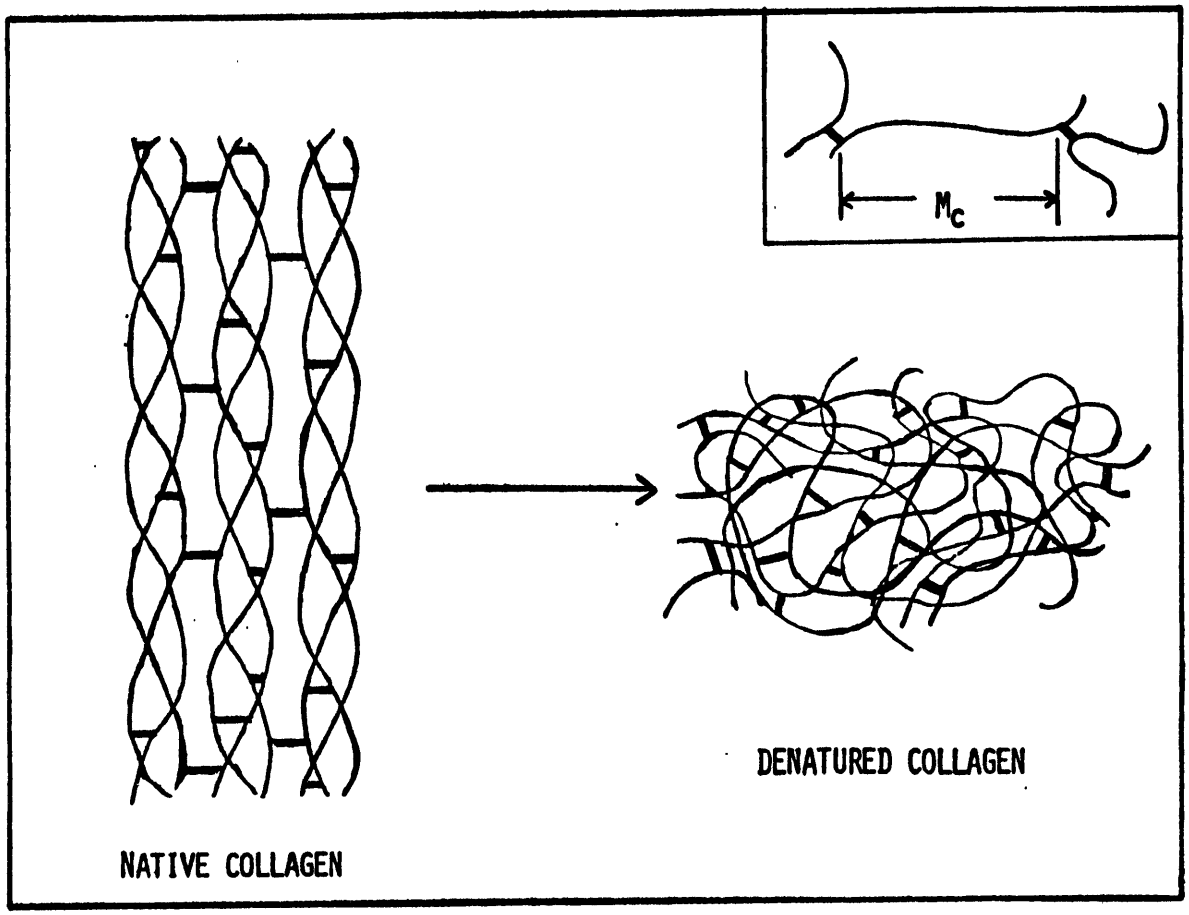


Fig. 3.9. Illustration of denaturation of a crosslinked collagen fiber. The meaning of the number avearge molecular weight of the chain (M_c) is illustrated in the inset. (Huang, 1974)

Figure 20

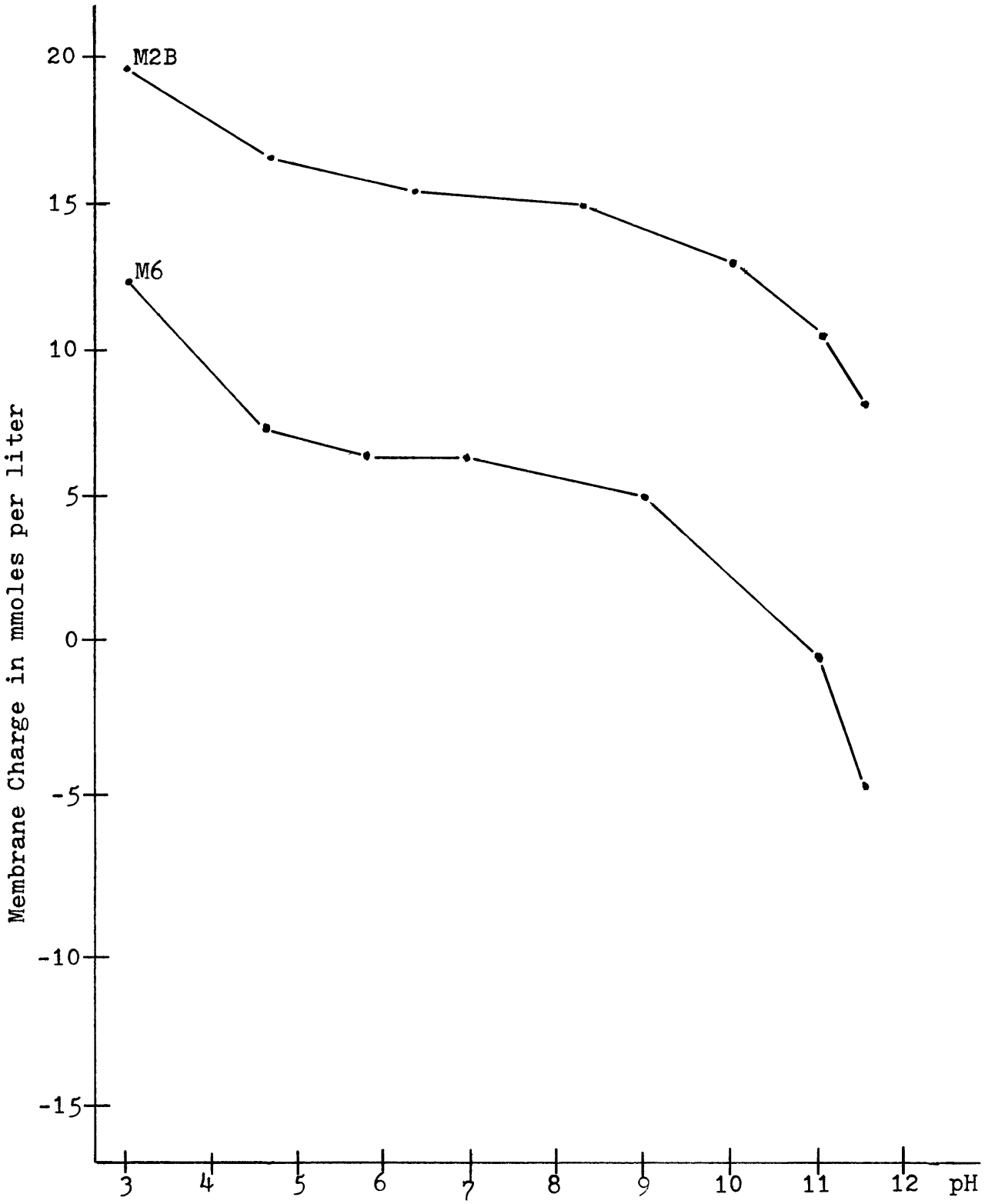
of collagen changes significantly and irreversibly upon exposure to a pH of less than 5 or greater than 9. Whether the conformational changes primarily affect the glutamic and aspartic acid carboxyl groups or the arginine side chains, or whether both are affected, cannot be adequately determined from the experiments done in this study. Experiments could be performed utilizing this technique to indicate the relative involvement of the groups, however. It would be necessary to study the charging behavior of the membranes at higher pH than was done in this study, namely pH 12 to pH 13. In that regime the arginine groups would deprotonate and the effect on the hysteresis would determine the relative involvement of the arginine groups. As was mentioned in the experimental section, however, the ionic strength and activities are assumed to remain constant over the course of the experiment. That assumption was valid only due to the limited pH range. At pH 12-13 radical changes would occur in both parameters. Either more concentrated salt solutions would have to be employed in that regime or corrections would have to be included in the calculation of the volume charge. Another problem might be partial denaturation of the collagen.

Since no more hysteresis was observed after pH 3 was reached, the analysis which follows will only consider the experimental data corresponding to the measurements taken

after reaching pH 3. As long as such a practice is consistently followed, it seems possible to draw comparisons between membranes studied by this technique.

Figure 21 shows only the CD branches for membranes M2B and M6. It is apparent that the membrane containing 5% chondroitin-6-sulfate is consistently more negatively charged than the pure collagen membrane. At pH 3 it is 7.2 mmoles/liter more negatively charged, while at pH 7 the difference is 10 mmoles. Those observations are explained by the previously detailed charge properties of C-6-S. At pH 3 the MPS sulfate group contributes one negative charge per subunit, while at pH 7, the carboxyl groups deprotonates and the MPS thus contributes more negative charge per subunit.

The actual amount of MPS present in M6 cannot be easily determined from this experimental data. However, the more relevant comparison to make between membranes is not the amount of MPS added per se, but the effect the MPS has on the characteristics of the membrane. The characteristics such as swelling ratio, mechanical strength, adhesion to wounds, resistance to enzymatic degradation, and permeability to ions are the important parameters. Thus the measurement of the effective charge density, which strongly influences many of the above properties, is nonetheless an important quantity to measure. Correlation



Membrane Charge of M2B and M6 as a Function of pH.

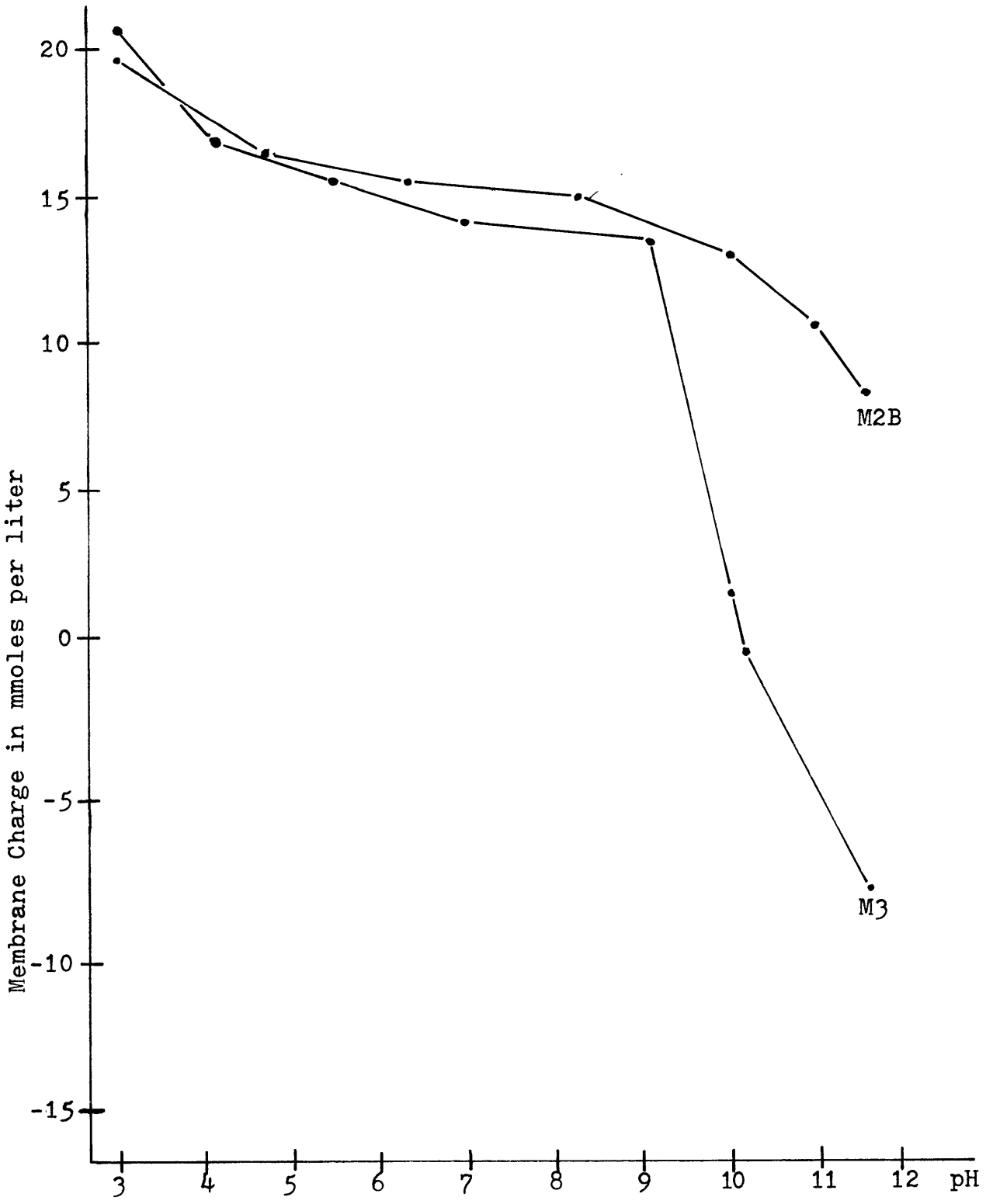
Figure 21

of such parameters as the in vivo behavior of implanted membranes with the charge densities measured by this technique therefore seems a useful adjunct to correlation with the content of MPS.

Figure 22 compares M3 to the pure collagen membrane M2B. M3 contained approximately 4% MPS, but was cross-linked at acid pH. The difference in charge character is quite significant. M3 doe not begin to appear more negatively charged than pure collagen until pH 9. Thus, in the physiological pH region near pH 7.4, M3 exhibits a a charge nature much more like collagen than does M6.

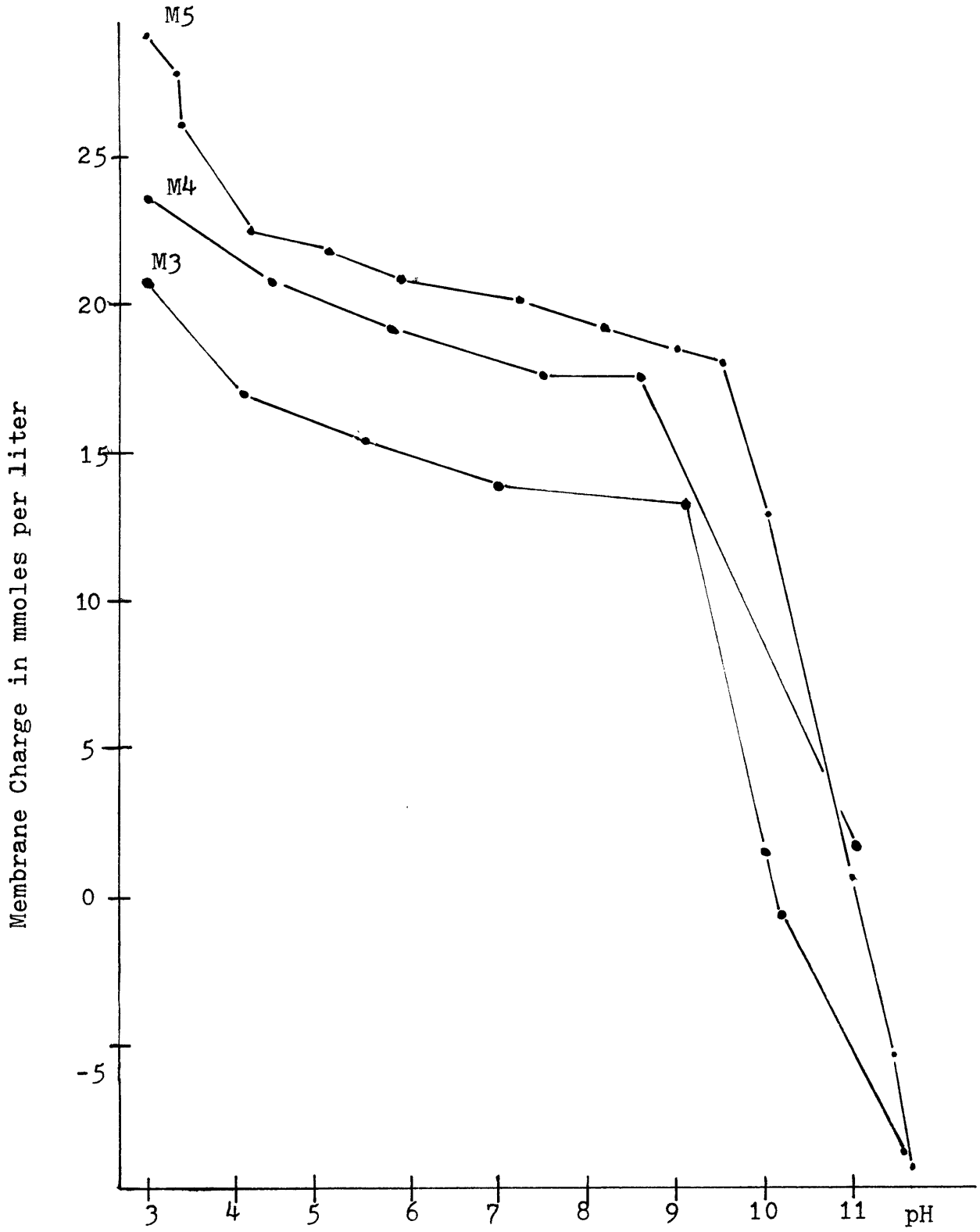
Quite recent implantation studies appear to indicate that membranes similar to M6 show greater promise as skin replacement materials than do membranes like M3 (Yannas,1976). Thus, although the MPS content appeared to be the same, the charge density was quite different, as was the in vivo behavior. There have been other indications that membranes which are negatively charged under physiological conditions have better in vivo characteristics than positively charged ones. (Yannas,1976) Therefore, volume charge density measurements seem to be useful correlates of in vivo behavior experimentally as well as theoretically.

Another useful comparison to make is between the extent of crosslinking and the charging characteristics of a membrane. Figure 23 compares membranes M3, M4, and



Membrane Charge on Collagen(M2B) and Collagen-MPS Composite(M3) as a Function of pH.

Figure 22



Membrane Charge of Collagen-MPS with Varying M_c.

Figure 23

M5, which differ only in the extent of crosslinking. As detailed previously, for membranes crosslinked in glutaraldehyde, lysine is apparently primarily involved in the crosslinking process. A heavily crosslinked membrane should therefore appear more negatively charged than a lightly crosslinked one.

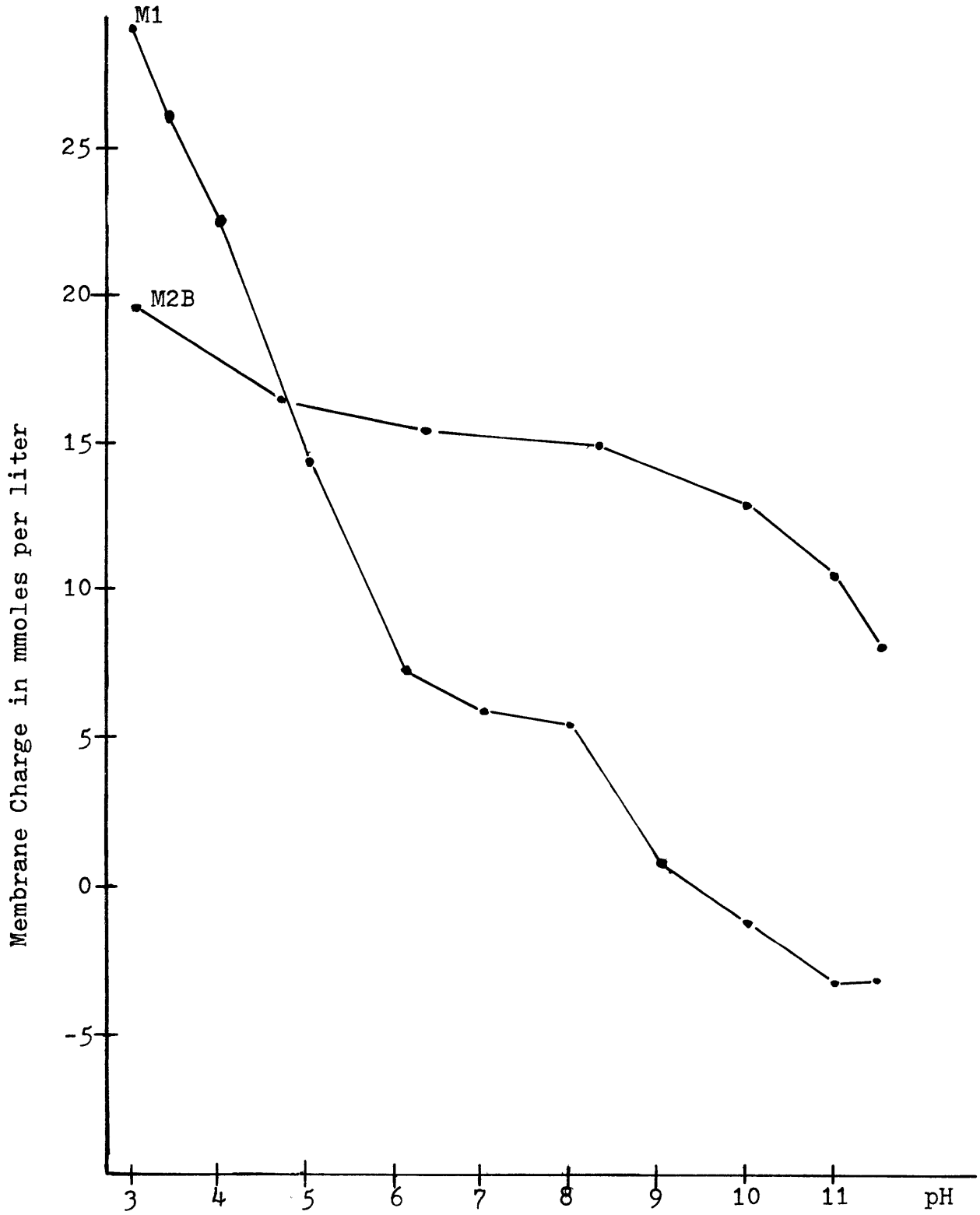
The number average chain molecular weight, M_c, is a standard measure of crosslink density in biological molecules. As pictured in Figure 20, M_c is the average gram molecular weight of the molecular segments between crosslinks. A very low M_c thus means very heavy crosslinking. For M3, M_c was 4000, for M4, 15500, and for M5, 24000. As can be seen, there is a very good experimental correlation between crosslink density and the volume charge measured. In fact, if the crosslink density of M4 were unknown, it could be interpolated from the graphs of M3 and M5 shown in Figure 23. In the pH 5 to pH 8 region M5 is about 6 mmoles per liter more positive than M3 while M4 is about 4 mmoles more positive. Thus the M_c of M4 could be interpolated as 4000 + (4÷6)(24000-4000) or about 17000. This calculated value is within about 15% of the measured value.

It may also be noted that the charge difference between M3 and M5 disappears about at pH 10-11. Since the difference in charge is due to the elimination of

lysine amino groups in the crosslinking process, the charge difference should disappear in a region where lysine would normally be uncharged. Since the pKa of lysine is about 10.5, the experimental observation seems to be explained. If the involvement of lysine in the crosslinking process had not been known, it would have been implied by this data. Many other such chemical reactions in biological molecules could perhaps be studied in the same manner. The method outlined here could very often narrow the possibilities in cases where charged groups are created or destroyed by the reaction.

A comparison can usefully be made between a pure collagen membrane crosslinked at acid pH, M2B, and a pure collagen membrane crosslinked at neutral pH, M1. In Figure 24 the experimental data is reproduced. Membrane M1 appears to be more positively charged than M2B until pH 5 is reached, then it appears much more negative. Since pH 5 is the approximate region in which carboxyls deprotonate, this behavior may be related to the carboxyls. If the membrane crosslinked at neutral pH has many more accessible carboxyl groups and somewhat more accessible basic groups, the observed behavior can be explained as follows.

At pH 3, the carboxyl groups are uncharged and M1 is somewhat more positive due to its larger number of basic groups. As the pH goes above pH 5, however, and the



Membrane Charge of Collagen Crosslinked at Neutral and at Acid pH.

Figure 24

carboxyl groups deprotonate, M1 looks much more negative due to its preponderance of carboxyl groups.

The experimental data from membrane M2A showed little or no variation in charge with pH. Visual examination of M2A showed the presence of a large number of small entrapped air bubbles in the membrane. It seems probable that several of the bubbles extended completely through the membrane, producing small holes. The presence of such holes would explain the anomalous behavior. The majority of the diffusion of ions would take place through the holes rather than through the pores in the membrane. Therefore the charge of the holes instead of the charge of the membrane was being measured. Ideally that charge should be zero, but here it was slightly negative.

In conclusion it seems that this technique offers a unique and valuable method of studying polyelectrolyte materials in the solid state. Many things can be inferred concerning subtle conformational changes, and in chemical reactions where charged groups or ionizable groups are involved, the groups involved can be pinpointed or the field of possibilities narrowed.

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