FACTORS AFFECTING THE THROMBIN INHIBITING ACTIVITY OF HEPARIN WHEN IMMOBILISED TO HYDROGELS BY COVALENT BONDING

BY

SEW-WAH TAY

B.S. FOOD SCIENCE AND TECHNOLOGY,
UNIVERSITI PERTANIAN MALAYSIA (1980)

S.M. FOOD SCI., MASSACHUSETTS INSTITUTE OF TECHNOLOGY (1983)

S.M. CHEM. ENG., MASSACHUSETTS INSTITUTE OF TECHNOLOGY (1983)

SUBMITTED TO THE DEPARTMENT OF
APPLIED BIOLOGICAL SCIENCE
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF SCIENCE

AT THE

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

May 12,1986

Signature of Author_____
Department of Applied Biological Science
May 12,1986

Certified by_____
Professor E.W. Merrill
Thesis Supervisor

Accepted by_____
Prof. S. Tannenbaum
Chairman, Departmental Graduate Committee

FACTORS AFFECTING THE THROMBIN INHIBITING ACTIVITY OF HEPARIN IMMOBILISED TO HYDROGEL BY COVALENT BONDING

by

SEW-WAH TAY

Submitted to the Department of Applied Biological Sciences on May 12, 1986 in partial fulfillment of the requirements for the Degree of Doctor of Science

ABSTRACT

Heparin was covalently attached via tresyl chloride to hydrogels synthesized by radiation crosslinking. The activity of the bound heparin as determined by its ability to inhibit thrombin depends on the ability of the heparin molecule to freely assume a conformation similar to that when in free solution.

For hydrogels made from poly(vinyl alcohol) with its numerous hydroxyls the specific activity of the immobilised commercial heparin depends inversely on the degree of activation of the hydroxyl groups. The tightly bound heparin lost almost all of its initial activity. Higher yields of bound heparin were obtained when coupling was carried out at 22^{o}C compared to 4^{C}.

Heparin was modified by nitrous acid degradation at the N-unsubstituted glucoseamine at pH 4. The aldehyde generated at the point of cleavage was modified to carry a terminal amine group increasing the number of terminal amine on the heparin molecule by about 300% with only a 27% loss in activity and a 35% decrease in the average molecular weight. When coupled to activated PVA the retention of activity is more than 3 times that obtained when pure heparin was used. This is because the molecules were attached to the hydrogel solely via their amino terminals only and the hexadiamine provided an extra 6 carbon leash to the heparin molecules.

Spacing out the frequency at which the heparin was attached resulted in a ten fold increase in specific activity of the bound heparin. This was achieved by using Poly(ethylene oxide) hydrogel which have hydroxyls only at its terminus. The higher activity obtained with PEO was also partly attributed to the increase in leash length to which the heparin molecule is attached and the decrease in the incidence of multiple attachment of the molecule to the hydrogel.

Thesis supervisor : Prof. E.W. Merrill
Title : Carbon P. Dubbs Professor of Chemical Engineering

ACKNOWLEDGEMENT

The author would like to take this opportunity to thank Professor Edward Merrill for his technical and moral support throughout this project. His wonderful sense of humour and constant enthusiasm have made this project a pleasurable learning experience.

Many thanks also to Kenneth Wright at the MIT High Voltage Laboratory for his patience in operating the van de Graaf generator in between his tight schedule. Dr. Jack Lindon and other members of Dr. Salzman group especially Gerry, Helen and MaryAnn were very helpful in suggesting the various ways of testing the activity of the heparin. Prof. Langer, Prof.Salzman and Prof. Suter provided very useful suggestions in the way the thesis was developed.

This thesis would not have been completed in time without the assistance provided by Kathleen Dennison who maintained and upkept the Size Exclusion Chromatography instrument in the lab and also wrote the program that collected and analysed the data from the SEC. Other colleagues in the lab that have contributed to the stimulating intellectual atmostphere include Kyu-Ha Chang, Rick Pekala, Cynthia Sung, Betsy Robinson and Al Horn.

Rowena Sobarzo, Mona Wan and John Campbell in their capacity as UROP student have made significant contribution to the completion of this project. Rowena purified the nitrous acid modified heparin and John helped prepare the purified antithrombin. Mona assisted in the chemical analysis of the pure heparin.

The author is fortunate to have the constant support and encouragement from family members especially Ching Meng for his understanding and love, her mother-in-law for comming all the way here from Malaysia to help look after Ming-Hwei and Ming-Hwei for being such a wonderful and cooperative daughter.

This thesis was financially supported in part by a grant from the National Institute of Health.

To Ching-Meng and Ming-Hwei

TABLE OF CONTENTS

1. INTRODUCTION 15

2. LITERATURE REVIEW 17

2.1 HEMOSTASIS AND BLOOD COAGULATION 17

2.1.1 Platelet Activation 19

2.1.2 The Intrinsic Pathway 19

2.1.3 Extrinsic Pathway 22

2.2 Anticoagulant Action of Heparin 22

2.3 Heparin Molecule 28

2.4 Nitrous Acid Depolymerisation of Heparin 33

2.5 Enzymatic Depolymerisation of Heparin 36

2.6 Blood Surface Interaction 37

2.7 Heparinised Surfaces 39

2.8 Polyvinyl Alcohol (PVA) 43

2.9 Chemical Crosslinking Of PVA 45

2.10 Radiation Cross-Linking of Polymers 46

2.11 Effect Of Ionising Radiation On PVA 49

2.12 Radiation In Aqueous Solution 54

2.13 Immobilisation Techniques 58

2.13.1 Covalent Bonding Methods 58

2.13.2 Carbonyl Diimidazole 60

2.13.3 Sulphonyl Chlorides 63

3. OBJECTIVE 67

3.1 Specific Aims 67

3.2 Assumptions and Experimental system 68

3.2.1 Heparin 68

3.2.2 Hydrogel 68

4. MATERIALS AND METHODS 70

4.1 MATERIALS 70

4.1.1 Polyvinyl alcohol 70

4.1.2 Polyethylene oxide 70

4.1.3 Heparin 70

4.1.4 Chemicals 71

. 72

4.1.5 Antithrombin and Thrombin 72

4.1.6 Plasma 72

4.1.7 Radiation Facility 73

4.2 METHODS 73

4.2.1 Polyvinyl Alcohol solution 73

4.2.3 Measurements of Mc 76

4.2.4 Molecular weight determination 79

4.2.5 Protein Penetration Test 80

4.2.6 Heparin Penetration 81

4.2.7 Heparin Determination 83

4.2.8 Fluorescamine Assay of Free Amino Groups . 84

4.2.9 Determination of the Carboxyl Groups . . . 85

4.2.10 Amino Acid Analysis 85

4.2.11 Reducing Group Determination 86

4.2.12 Partial Nitrous Acid Degradation of Heparin 86

4.2.13 Amination of Heparin 87

4.2.14 High Voltage Paper Electrophoresis 89
4.2.15 Drying of the Hydrogel 89
4.2.16 CDI Activation 90
4.2.16.1 Determination of carbonyl imidazole on the gel 91
4.2.17 Activation by 2,2,2-Trifluoroethanesulphonyl Chloride . . . 93
4.2.18 Determination of Tresylates on the Hydrogel 94
4.2.19 Coupling of Heparin 94
4.2.20 Comparison between the reactivity of the hydroxyl and the amine group in the coupling 95
4.2.21 Stability of bound Heparin 96
4.2.22 Heparin activity in solution 97
4.2.23 Activity of Bound Heparin 98

6 RESULTS AND DISCUSSION 101
6.1 Crosslinking of PVA in solution 101
6.1.1 Molecular weight of PVA 101
6.1.2 PVA Hydrogels 103
6.1.3 Penetration of Globular Protein into the Gel 106
6.1.4 Heparin Penetration into the hydrogel . . . 110
6.2 Characterisation of Commercial Heparin 112
6.2.1 Commercial Heparin (PH and CPH) 112
6.3 Chemistry of coupling Reaction. 118
6.3.1 Coupling of Serine and Glycerol to PVA . . . 118
6.3.2 Stability of the C-N Bond 120

6.4 Coupling of Pure Heparin to PVA 120

6.4.1 Stability of Bound Heparin in Plasma 124

6.4.2 Effect of temperature on the Rate of Coupling of Heparin 127

6.5 Effect of the Degree of Gel Activation on Immobilised Heparin 137

6.6 Modified Heparin 140

6.6.1 Reductive Amination of Pure Heparin 140

6.6.2 Nitrous Acid Degraded Heparin 143

6.6.3 Coupling of Modified Heparin onto PVA 150

6.7 Coupling of Pure Heparin to PEO 154

9. REFERENCES 160

Appendix A 170

APPENDIX B 176

Appendix C 177

APPENDIX D 178

APPENDIX F 181

List of Figures

2-1: Intrinsic pathway of blood coagulation. 18

2-2: Extrinsic pathway of blood coagulation. 20

2-3: The sites of action of Heparin and Antithrombin-3 within the coagulation cascade. 25

2-4: The structure of heparin proteoglycan. 29

2-5: The structure of a typical hexasaccharide of heparin. 31

2-6: Structure of antithrombin binding sequence (Lindahl et al. 32

2-7: Stucture of PVA 43

2-8: Structure of PVA 43

2-9: Reaction of PVA and Borate 46

2-2: Effect of degree of polymerisation, concentration and temperature on the gelling of PVA by boric acid and borax (Finch, 1981). 47

2-10: Polymer Structure that degrade or crosslink when irradiated 48

2-11: Main chain rupture 51

2-12: Intramolecular dehydration 52

2-13: 100 eV yields of crosslinks [G(X)] and main chain scissions [G(S)] versus the mole fraction of poly-dimethyl siloxane in toluene 53

2-14: Primary Products of Irradiated water 55

2-15: Reactions involved in the irradiation crosslink of PVA 56
2-16: Coupling by CDI activation 61
3-1 : Schematic diagram of the hydrogel surface 69
4.1 :Schematic Diagram of the Radiation Setup at the High Voltage Laboratory at MIT 74
4-1: Hydrolysis of activation group 92
4-1 : Universal calibration curve for SEC 102
6-2: The interjunction number average molecular weight as a function of dose 104
6-3: $1/M_c$ versus dose 105
6-4: Concentration of Heparin in the supernatant as a function of time 111
6-5 : Stability of Bound ^{14}C-serine as a function of pH at 37 ^{o}C 122
6-6 : Leaching of Bound ^{14}C-serine into platelet rich plasma 123
6-7 : Degree of activation as a function of coupling reagent 125
6-8 : Stability of bound ^{35}S-Heparin in Platelet Rich plasma at 37^{o}C 126
6-9 : Rate of coupling of Heparin to PVA at 22^{o}C and pH 10 128
6-10 : Coupling of Heparin to PVA at 4^{o}C and pH 10 129
6-11 : Schematic diagram of the possible ways by which Heparin could be bound to tresylated PVA 134

6-12 :Total Heparin activity found in PVA gel versus time of coupling. 136
6-13 : Specific activity of the immobilised heparin on PVA with time of coupling 136
6-14 : Heparin bound as a function of Degree of Activation at 22oC and pH10 139
6-15 : Molecular weight distribution of Pure Heparin and Nitrous Acid degraded Heparin 144
6-16 : Molecular Weight Distribution of Nitrous acid degraded heparin before and after reacting with Hexadiamine. 147
6-17 : High Voltage Paper Electrophoresis of Heparin 149
6-18 : Schematic diagram of the binding of nitrous acid modified heparin onto tresylated PVA. 153
6-19 : Schematic diagram of heparin bound to PEO network 157
9-1 : Titration curve for Pure Heparin 177
9-2 : Swelling Ratio of CDI activated PVA in DMF 180
9-3 : Effect of levels of Imidazole substitution of PVA on swelling ratio 181
9.4 : Schematic Diagram of a ramdomly crosslinked network . 181

Lists of Tables

6-1: Penetration of protein into PVA hydrogels 107

6-2: Average Distance between junctions in PVA hydrogels 109

6-3 : Heparin Preparations 113

6-4 : Amino acid composition of Whole Heparin 115

. 116

6-5 : Chemical Analysis of Diosynth Heparin 118

6-6 : Coupling yield of ^{14}C-Serine and ^{14}C-Glycerol to PVA 120

6-7 : Rate of Coupling of Heparin to PVA at 22°C and pH 10 132

6-8 : Rate of Coupling of Heparin to PVA at 4°C and pH 10 132

6-8 : Degree of Activation of PVA with Tresyl Chloride in DMF at 22°C

. 138

6-9 : Coupling of Heparin as a function of Degree of activation with tresyl chloride at 22°C and pH 10 139

6-10 : Reductive Amination of Pure Heparin 141

6-12 : Effect of Nitrous Acid degradation and Reductive Amination of Heparin on the reducing group and primary amine content

. 148

6-13 : Nitrous Acid Heparin bound to PVA 151

6-14 . Binding of Heparin to PVA versus PEO Hydrogel 156

1. INTRODUCTION

In view of the ever increasing demand for polymeric biomaterials which can be applied to a variety of cardiovascular systems, considerable attention and research have been placed on the development of blood compatible materials.

Thrombosis induced by contact with artificial materials is still the major obstacle to further development of artificial internal organs. It accounts for much of the problems faced when blood is circulated through an extracorporeal system such as cardiopulmonary bypass or hemodialyser. At present, blood coagulation in these systems are inhibited by the addition of the anticoagulant, heparin, into the blood. This however, gives rise to undesirable side effects namely, thrombocytopenia (Ansel et al.,1980; White et al.,1979) and skin necrosis in subcutaneous injections and internal bleeding.

A more satisfactory way to achieve non-thrombogenicity would be to have heparin immobilised on the surfaces of biomaterials that are themselves relatively passive towards plasma proteins and blood platelets since both plasma proteins and blood platelets contribute to the formation of a thrombus.

However when heparin was bound to various substrates it has been found to lose most if not all of its antithrombotic activity. One of heparin's most important function is to accelerate the deactivation of thrombin by antithrombin-111

In general it has been found that amorphous, hydrophilic polymers have better compatibility with blood than crystalline and/or hydrophobic polymers. Some examples of the hydrophilic amorphous materials are polyethylene oxide(PEO), polyvinylalcohol(PVA)and polyhydroxyl ethyl methacrylate(PHEMA). PVA has secondary alcohols on every other carbon on its chains PEC has only terminal primary hydroxyls and PHEMA which has primary hydroxyls on every other carbon on the main chain backbone.

2. LITERATURE REVIEW

2.1 HEMOSTASIS AND BLOOD COAGULATION

When blood comes into contact with a foreign surface, tissue cells or collagen fibres a series of reaction occurs in the blood plasma in respond to these stimuli. The hemostatic mechanism of the body is set into motion with the final production of a hemostatic plug or thrombus that is made up of one or more of the following blood components viz blood platelets; whole blood cells and a network of fibrin polymer in which the former two can be embedded.

There are three possible routes to the formation of blood thrombi:

(a) The adhesion and aggregation of platelets at the foreign interface, leading to the formation of a platelet thrombus on that surface.

(b) The intrinsic blood coagulation system involving a series or cascade of enzymatic activation steps beginning with the surface activation of a glycoprotein, factor XII and ending with the formation of fibrin at the surface (Figure 2-1).

(c) The extrinsic blood coagulation system, initiated

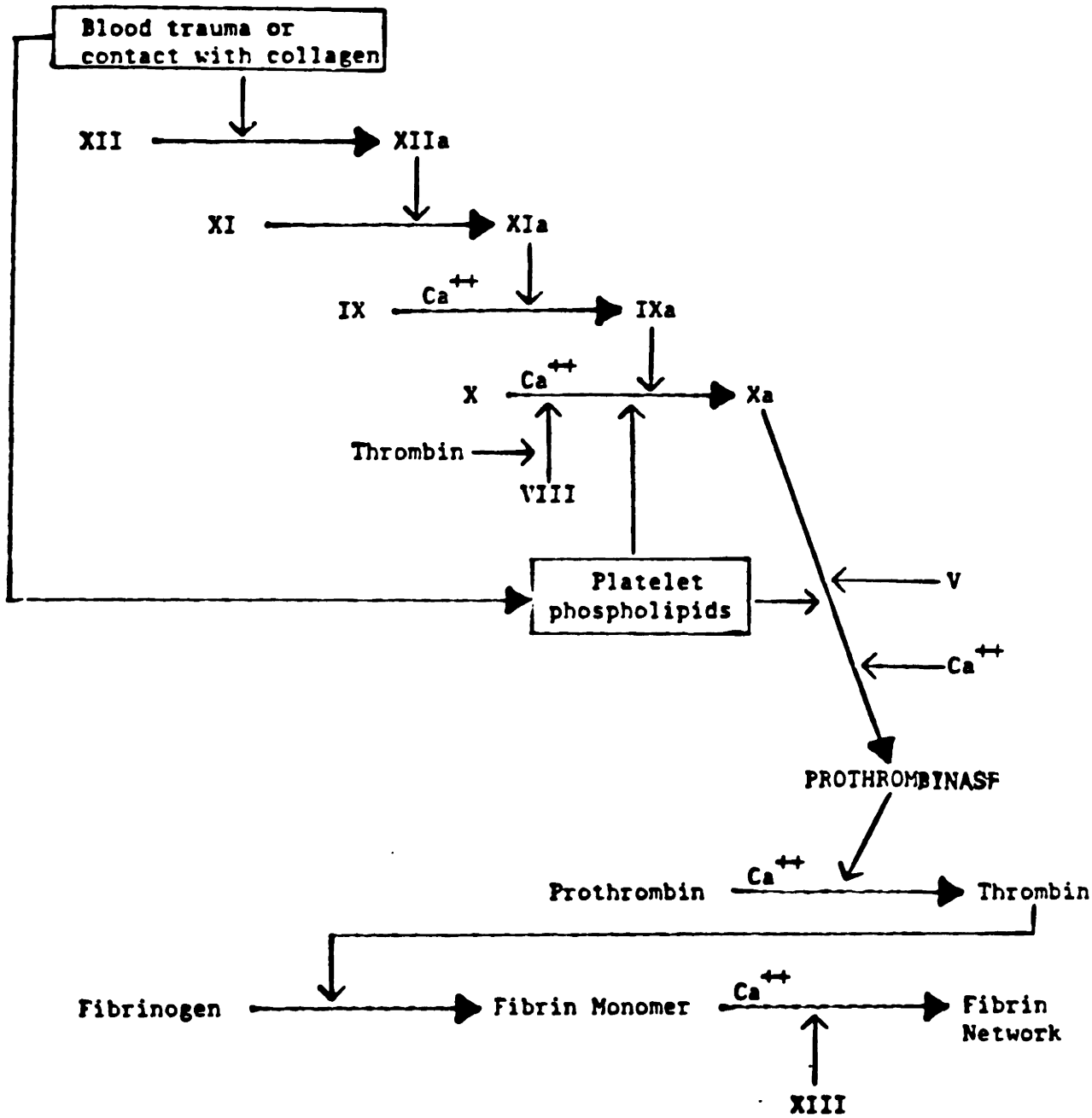


Figure 2-1: Intrinsic pathway of blood coagulation.

by the release of a tissue factor (thromboplastin) that can trigger a cascade of enzyme reactions similar to the intrinsic system and again leading to the formation of fibrin at the surface (Figure 2-2).

2.1.1 Platelet Activation

Platelets are normally present in human blood at a concentration of $2X10^8$ per ml. They are formed from the fragmentation of megakaryocytes in the bone marrow (Gordan and Milner, 1976) and in vitro has a life span of about 10 days. A protein called von Willebrand factor (a polymer of factor VIII) activates the platelets when a foreign surface is encountered by the blood. The activation process causes the platelets to assume irregular shapes and to become "sticky" so that they start to adhere to that surface.

These platelets then releases large quantities of ADP, cAMP, calcium, serotinin, 5-hydroxytryotamine(5HT), and prostaglandins all of which accelerate the formation of thrombaxane A in the plasma (Gordan and Milner,1976). ADP and thrombozane A in turn activate other platelets and cause them to aggregate with themselves and the already adherent platelets forming a porous thrombosis.

2.1.2 The Intrinsic Pathway

The intrinsic mechanism begins with the activation

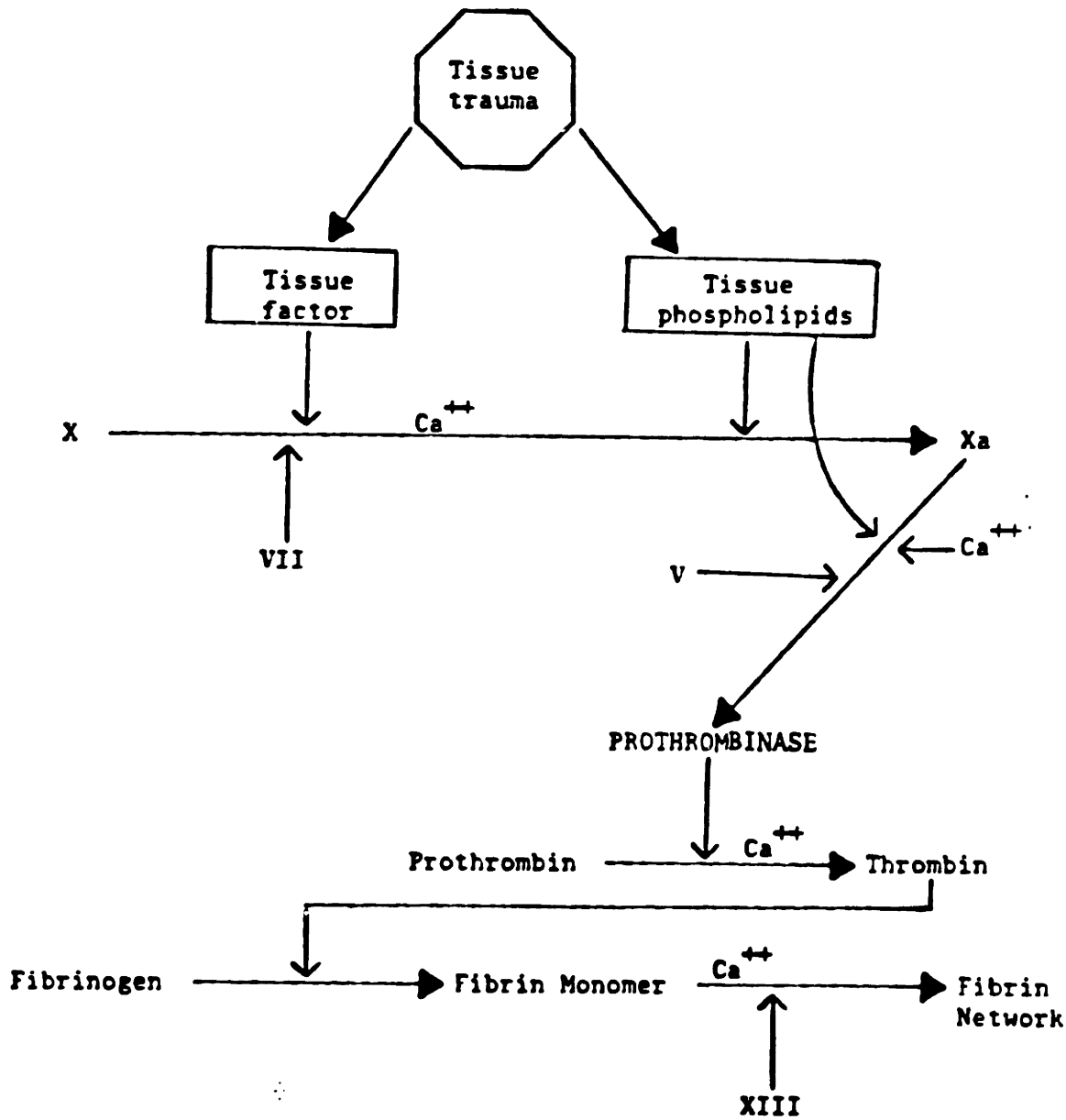


Figure 2-2: Extrinsic pathway of blood coagulation.

of factor XII and high molecular weight kininogen (HMK). Their adsorption to the surface induce a sequence of enzymatic reactions whereby the zymogens are converted to their respective enzymes. Traumatised platelets induce a phospholopid, platelet factor III (PF3) which together with kallikrein and prekallikrein are responsible for the cleavage of factor XII to factor XIIA. Factor XIIA in turn is a potent enzyme which is able to catalyse the hydrolysis of prekallikrein to kallikrein. This cyclic positive feedback reactions results in the formation of a avalanche of factor XIIA in the vicinity of the thrombotic surface within a short time. Factor XIIA catalyses the hydrolysis of XI to XIA. Factor XIA in conjunction with PF3 is responsible for activating factor X to XA. Factor XA together with factor V and platelet phospholipid in the presence of Ca^{++} form prothrombinase. The latter converts prothrombin to thrombin. Thrombin then cleaves four arginine-glycine peptide bonds in fibrinogen to form fibrin. The fibrin monomers then aggregrate to form a network entrapping the aggregated platelets and/or blood cells. Activated factor XII (fibrin stabilising factor) found in plasma and also released from the entrapped platelets causes crosslinking between adjacent frbrin threads to form an insoluble network (Guyton,1981).

2.1.3 Extrinsic Pathway

The extrinsic pathway is initiated when blood comes in contact with collagen or extravascular tissue. Tissue factor (factor 3) and tissue phospholipids are released into the blood. Tissue factor complexes with factor XII and in the presence of tissue phospholipid and calcium ion activates factor X to factor XA. Factors XA, V and the released tissue phospholipids form the complex prothrombinase (Hanahan et al., 1969). From here on the enzymatic cascade is identical to that of the intrinsic pathway. Table 2-1 list most of the clotting factors designated by both a roman numeral and a common name

2.2 Anticoagulant Action of Heparin

Six of the eleven proteins [i.e. prothrombin and factors VII, IX, X, XI and XII] that are involved in the coagulation system are converted to serine proteases. Antithrombin-3, normally found in plasma at a concentration of 150-250 ug/ml (Fougnot et al. 1984) inhibits the action of all six of these proteases to varying extent (Jordan et al., 1980).

Heparin, a mucopolysaccharide, acts as an anticoagulant by accelerating the action of antithrombin-3 many times. Rosenberg (1982) postulated that the mechanism of heparin's activity lies in the binding of antithrombin-3 at its lysine

Table 2-1 : Nomenclature for Blood Clotting Factors

CLOTTING FACTOR	COMMON NAME
I	Fibrinogen
II	Prothrombin
III	Tissue thromboplastin
IV	Calcium
V	Proaccelerin; labile factor; Ac-globulin; Ac-G
VII	Serum prothrombin conversion accelerator; SPCA; convertin; stable factor
VIII	Antihemophilic factor; AHF; antihemophilic globulin; AHG; antihemophilic factor A
IX	Plasma thromboplastin component; PTC; Christmas factor; antihemophilic factor B
X	Stuart factor; Stuart-Prower factor; antihemophilic factor C
XI	Plasma thromboplastin antecedent; PTA; antihemophilic factor C
XII	Hageman factor; antihemophilic factor D
XIII	Fibrin stabilizing factor

site. He came to this conclusion from observing that the heparin-antithrombin complex will decouple in 1M NaCl solution. This suggest that the binding force is mostly ionic in nature. Thrombin (or the other serine protease) is deactivated by the extremely reactive antithrombin heparin complex as schematised in figure 2-3. The resulting thrombin-antithrombin complex is released from the heparin, which is then free to activate other antithrombin-3 molecules.

A large amount of evidences indicated that antithrombin-3 undergoes a conformational change when it binds to heparin Nordenman and Bjork (1978) compared the UV-CD spectrum of heparin bound antithrombin-3 and free antithrombin. No changes in the far-UV CD spectrum of antithrombin was observed indicating very little changes in the secondary structure of antithrombin when bound to heparin. However, significant changes are observed in the near-UV absorption and CD spectra of antithrombin, suggesting a local perturbations of the environment of some aromatic amino acids of the protein when bound to heparin. A much larger change was seen for high affinity heparin than for low affinity heparin. This is consistent with the results obtained when a tryptophan residue in antithrombin was modified (AT*) (Karp et al., 1984). The affininty for heparin by the modified AT3* was decreased by ten times but

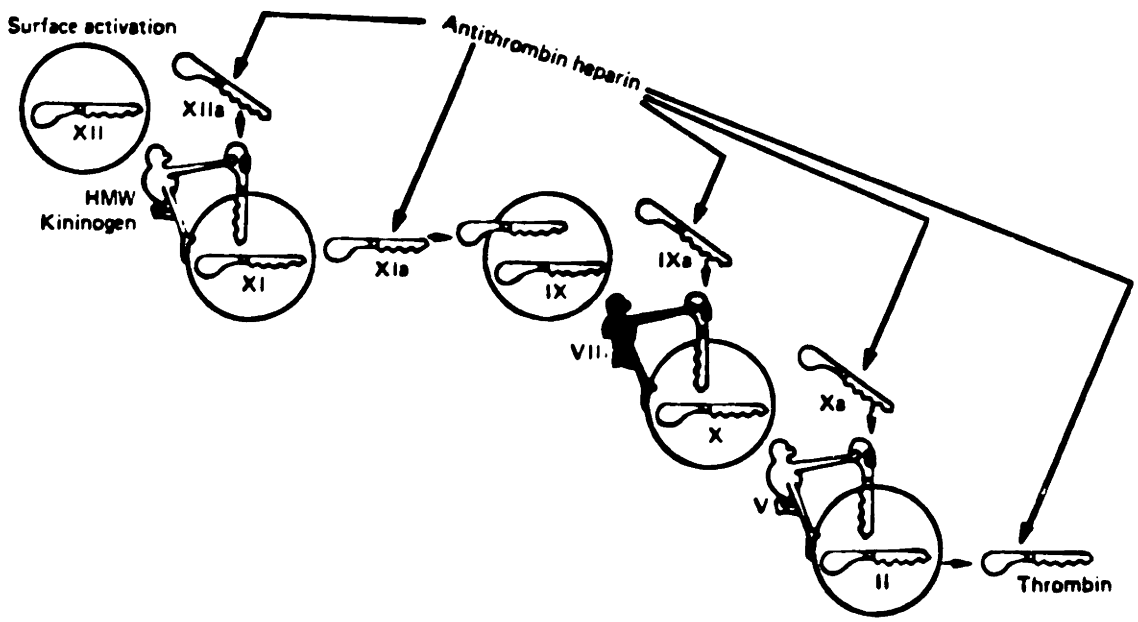


Figure 2-3: The sites of action of Heparin and Antithrombin-3 within the coagulation cascade.

the ability of the heparin-AT3* complex has less than one hundredth of the original activity to deactivate thrombin. The AT3* - thrombin interaction on the other hand, was not affected by the modification. This implies that the tryptophan residue is involved in the conformational change in the antithrombin when bound to the heparin and that this change is essential for the acceleration of the antithrombin activity.

Although heparin's activity have been studied mostly in terms of antithrombin binding to it, there are evidences to indicate that thrombin (and possibly the other proteases) also complex with heparin.(Jordan et al.,1979; Griffin, 1983). Some researchers (Sefton and Goosen,1981; Griffin,1981) believe that the binding of heparin to thrombin constituted the first step in the mechanism of heparin activity. Significant inactivation of thrombin by immobilised heparin occur only when thrombin was added to immobilised heparin before antithrombin. The possibility that the loss of thrombin activity could be due to adsorption of the protein to the material was not discussed.

2.3 Heparin Molecule

Due to its anticoagulant activity, the structure of heparin has been extensively studied. Heparin is a highly sulphated mucopolysaccharide with a molecular weight of 3,000 to several million in its natural form. It is found in all animals above the level of the horseshoe crab family, and is distributed throughout a variety of organs in the mammalian species (Engelberg, 1963).

In nature, macromolecular heparin consists of a protein backbone with extended sequence of alternating glycine and serine residues. Virtually all of the serine residues bear a heparin polysaccharide chain connected by a short linkage region in a comb-like arrangement as depicted in Figure 2-4 (Rosenberg, 1982).

The individual mucopolysaccharide chains have molecular weights of 60,000 to 100,000 daltons. In the extraction and purification steps, the protein backbone is digested and the "comb" cleaved. Cleavage also occur along the mucopolysaccharide chain, giving the familar commercial heparin with a broad distribution of molecular weights in the range of 5,000 to 20,000. Some of these molecules might bear terminal serine if the manufacturing process was mild.

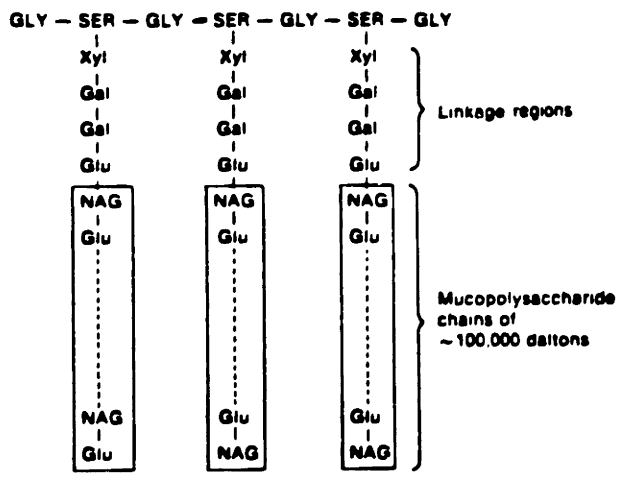


Figure 2-4: The structure of heparin proteoglycan.

The repeat unit in heparin is a disaccharide of glucoseamine and uronic acid linked by alternating α-D and β-D (1-4) bonds (Jeanloz, 1975). The uronic acid groups may exist as iduronic acid or glucoronic acid. The hydroxyl, carboxyl and amino groups may or may not be acetylated or sulphated. Figure 2-5 shows a typical hexasaccharide of heparin. Detailed analytical data indicated that the dissacharide unit, iduronsyl-2-sulphate- glucoseamine-2,-6-disulphate, represents the major structural element of all heparin preparations (Karapally and Dietrich,1970).

A variety of physical methods have been used to study the 3-D conformation of porcine heparin. It appears that in solution, the heparin exists as a loose helical coil with the sulphate and carboxyl groups at the peripheral and pointing outwards (Stivala et al., 1967).

Lindahl et al. (1981) and Rosenberg and Lam (1979) demonstrated by antithrombin affinity chromatography on nitrous acid cleaved heparin, that only a fraction of the heparin molecule binds to antithrombin-3. Lindahl et al. (1981) showed that the shortest high affinity oligosaccharide that will bind tightly to antithrombin-3 is an octasaccharide

with the following structure shown in figure 2-6.

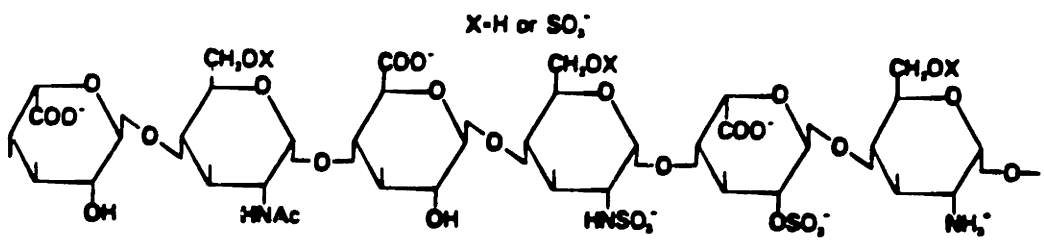
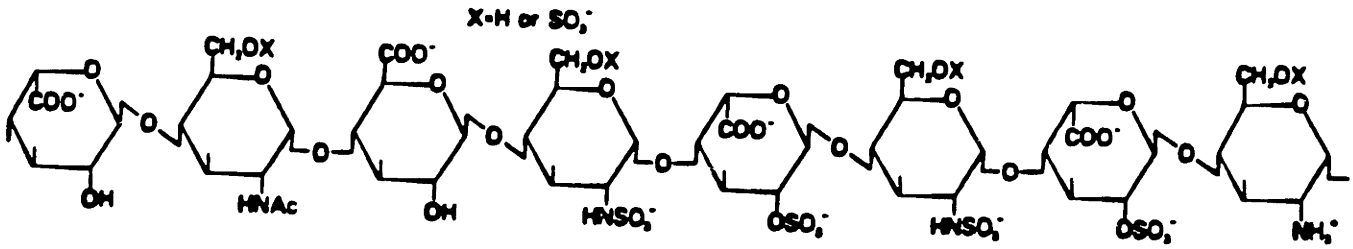


Figure 2-5: The structure of a typical hexasaccharide of heparin.

Figure 2-6: Structure of antithrombin binding sequence (Lindahl et al., 1981).



Rosenberg and Lam (1979) postulated that the antithrombinbinding sequence is the tetrasaccharide numbered 1 to 4, while Lindahl et al. (1981) suggested that the antithrombin binding sequence is contained within the pentasaccharide numbered 2 to 6. It must be noted that, although the antithrombin binding sequence is crucial to anticoagulant activity, the expression of such activity also depends on other regions of the heparin molecule (Laurent et al., 1978; Holmer et al., 1982). The other regions of the heparin are required presumably to bind to the proteases in order that the antithrombin can deactivate them. These two regions [i.e. the antithrombin and the protease binding sequences] may be widely separated on the heparin molecule, which then requires the heparin molecule to be able to freely assume the conformation that would bring the two regions close to each other in order that anticoagulant activity may be expressed. This may be the primary reason why heparin bound to the surface generally tend to have a much lower activity than that found in solution.

2.4 Nitrous Acid Depolymerisation of Heparin

Nitrous acid cleavage of polymers containing 2-amino-2-deoxy-D-glucosidic bonds have been reviewed by Horton and

Philips (1973). The cleavage is initiated by nitrosation of the free amino group followed by the loss of nitrogen with a ring contraction of the D-glucoseamine residue to 2,5-anhydro-D-mannose coupled to the elimination of tha aglycone. The yield of the anhydro-mannose depends upon the 2-amino-2-deoxy-D-glucoside involved (Shively and Conrad, 1970; Erbing et al., 1973) and the reaction conditions (Lagunoff and Warren, 1962).

Nitrous acid is usually generated in situ by the reaction of inorganic nitrite salt with an acid. Some formulation of nitrous acid will cleave only the D-glucoseamine glucoside when the amino group is unsubstituted (Lindahl et al.,1973) while others will cleave the glucosides of N-sulphated-D-glucoseamine only (Cifonelli,1968). The results of the cleavage is primarily anhydro-mannose which then becomes the new reducing terminal of the polysaccharide. Shively and Conrad (1976) did a very careful study of the effect of pH on the depolymerisation of heparin by nitrous acid. N-substituted heparin will depolymerise efficiently only at pHs less than 1.5 while completely desulphated heparin will not. The reverse is true at pHs between 3 and 4; 90% of glucoseamines in N-desulphated heparin were cleaved to anhydromannose while only 13% of the glucoseamines from the normal heparin were affected after 10 minutes of reaction time at room temperature. Both the N-sulphated and N-desulphated amino sugars give the same

products in identical yield indicating that both reactions involve a common nitrosated D-glucoseamine intermediate (Shively and Conrad, 1976). The nitrosating species involved is not nitrous acid itself but a product formed from it and these are different at different pHs. The two species involved are nitrous acidium ion (H_2ONO^+) and N_2O_3 formed by the following sequence of reactions:

$$H^+ + HONO \longrightarrow H_2ONO^+ \qquad (1)$$

$$HONO \xrightarrow[pka=3.35]{} H^+ + NO_2^- \qquad (2)$$

$$H_2ONO^+ + NO_2^- \rightleftharpoons N_2O_3 + H_2O \qquad (3)$$

The formation of N_2O_3 is catalysed by carboxylate ion and the optimum condition for its formation occur in the pH range of 2.5 to 4.0 where both H_2ONO^+ and NO^{2-} are present in relatively high concentration. At higher pHs the concentration of nitrous acidium ion is low and limits the rate of formation of N_2O_3. At lower pHs the low concentration of NO_2^- becomes rate limiting. At pH 4 the rate of formation of N_2O_3 is highest (Hughes et al., 1957) and the rate of formation of anhydromannose from glucoseamine is also the maximum (Shively and Conrad,1976). The nitrosating species for N-desulphated glucoseamine was thought to be most probably the N_2O_3 species.

N_2O_3 does not attack N-sulphated glucoseamine and hence the latter is deaminated at pH below 1.5 where the concentration of the former is negligible. The nitrous acidium ion

(reaction 1) is the main nitrosating reagent in this case. The pH dependence of nitrous acid degradation of heparin have allowed reseachers to selectively depolymerise heparin and elucidate the sequence of the heparin molecule.

2.5 Enzymatic Depolymerisation of Heparin

The enzyme heparinase can be purified from bacterium sources, specifically Flavobacterium heparinium (Linker and Hovingh, 1972). Its enzymatic property have been employed to remove heparin from blood after undergoing heparin therapy in kidney dialysis system (Langer et al., 1982) to reduce the side effects of the heparin therapy. Heparinase-like activity have been reported in human platelets (Oldberg et al., 1980; Oosta et al. 1982) and in mouse mastocytoma cells (Thunberg et al., 1982). The heparitinase in platelets is an endoglucosidase present within platelet lysosomes (Oosta et al., 1982). When isolated and purified it can cleave heparin-like substrate at the glucuronsyl-glucoseamine linkages when the amine group is sulphated.

Its ability to degrade heparin in vivo is questionable since the clearing of heparin in blood would otherwise occur at a much greater rate than is normally observed. Oldberg et al., (1980) put forward a theory that the heparitinase in platelets are stored in lysosomes and is released only after induction of the platelet release reaction. The platelet

release reaction required to release the enzyme appear to be when platelets adhere to a surface and not when they aggregate since endothelial extracellular heparan sulphate was degraded by platelet only after platelets began to spread on the surface (Yahalom et al., 1984).

2.6 Blood Surface Interaction

All biological implants and devices that come in contact with blood are recognised by blood as foreign. This results in the formation of a thromboemboli. Various researchers have put forward a number of hypotheses with respect to the mechanics of blood coagulation at foreign surfaces. Surface composition, water sorption, surface crystallinity, surface roughness, mechanical compliance of the surface and the bulk, are some of the properties that have been studied in relation to the suitability of the materials as a biomaterial (Merrill, 1977; Bruck, 1974; Berger and Salzman, 1974).

Materials with a critical surface tension (γ_c) between 20 to 30 dyne/cm are generally less thrombogenic than those with below or above this range (Baier et al., 1968). This however, is not the only determinant as some polymers such as poly(vinylfluoride) and poly(trifluoroethylene) has γ_c in this range but are still very thrombogenic (Salzman and Merrill, 1982).

Materials that swell in water have been known to exhibit good compatibility with blood due to the lower free energy of the hydrated interface. The swollen material is postulated to present a diffused surface to the blood and is more "fluid" (Suzuki et al., 1981). The diffused mobile chains of the polymers at the surface decrease protein adsorption and hence decrease protein and platelet activation. This decrease in protein adsorption at the hydrated interface can be attributed to the low polymer concentration at the interface and/or a decrease in interaction between the protein and the hydrophilic polymers.

Protein adsorption onto surfaces usually precede platelet activation. Fibrinogen when adsorbed onto a surface, undergoes a conformational change which exposes specific sites that platelets recognise (Vroman, 1981). Therefore, a good surface generally should have low fibrinogen adsorption and also should not change the conformation of the adsorbing protein.

Hard crystalline and glassy materials are thrombogenic as expected from the above discussion. The repeating order of the groups on the crystalline surface interact strongly with the adsorbed protein which then get denatured, exposing active sites on the protein which then set off one or more

of the three clotting systems.

In a series of studies with segmented ether polyurethanes (SPU), Merrill and Salzman (1983) had already shown that platelet retention and thrombin adsorption decrease as the percentage of polyethylene oxide (PEO) in the SPU increases. The hard crystalline segments in the SPU were found to be buried within the highly mobile, amorphous PEO as its fractions increased. Similar conclusion was reached by Pekala (1984) with his silicone - PEG material whereby the juctions are made of silicone molecules rather than diisocyanate material as in the urethanes.

The presence of heparin in the blood or on the surfaces of these relatively "good" materials should greatly improve their compatibility with blood.

2.7 Heparinised Surfaces

At present, patients undergoing surgery, kidney dialysis or have artificial organ inplants are given heparin intravenously or subcutaneously to prevent thrombosis. Thrombotic complications from long term heparin therapy have been reported. These include thrombocytopenia (depletion of blood platelets) (Ansel, et al., 1980), thromboembolism, skin and/or subcutaneous necrosis (White, et al.,1979) when

heparin was administered subcutaneously. Increase resistance to heparin therapy was also seen in some patients (Silver, et al., 1983). This gave the impetus to researcher to find ways of developing materials that will be compatible with blood indefintely.

Gott et al. (1961) was the first to ionically bind heparin to a graphite surface via a cationic detergent, benzalkonium chloride. They implanted a plastic ring of the heparinised material in the inferior vena cava of dogs and found that the ring remained essentially free of thrombus, as compared to a non-heparinised control ring. However, the non-thrombogenecity of this material is due to the slow release of heparin into the blood and hence is not suitable for long term contact with blood.

Covalent coupling of heparin, on the other hand, may cause the "denaturation" of the heparin resulting in only very limited thromboresistance (Grode et al., 1969; Halpern and Shirakawa, 1968). It is possible that at high immobilisation, the heparin is tied down to the surface along its length and hence loses its anticoagulant activity. Heparinised sepharose via cynogen bromide was found to bind a large amount of antithrombin but do not exihibit much activity with respect to thrombin -antithrombin complex formation (Lindon et al., 1985). These results support the idea that

both thrombin and antithrombin must bind to heparin at widely separated region and the heparin must be free to bring these two regions together.

Heparin anticoagulant activity decreases as the degree of carboxyl and hydroxyl derivitisation increase (Ebert et al.,1982) even in solution. This would imply that the two groups are important in determining its activity.

Merrill et al. (1970) developed a true heparinised polyvinylalcohol (PVA) hydrogel in which heparin is bound to PVA with glutaraldehyde via its hydroxyl groups. The clotting time of this material is several times longer than siliconised tubes or PVA coated tubes. Sefton and Goosen (1981) used ^{35}S-labelled heparin to check the elution of heparin from the above heparinised PVA. They proved that elution is negligible. The acetal linkage formed is however not stable at acidic pH (less than 5.8). The material moreover is structurally very fragile. Cholakis and Sefton (1983) postulated that the terminal serine residue is essential in grafting heparin onto a surface in order that the heparin may retain most of its initial activity. They found that heparin preparations that contain significant serine groups retain their activity better when bound. One possible reason for this is that the serine group allowed heparin to be bound to the surface at its terminal and hence the molecule is free to assume various conformation in the

solvent phase. Dincer (1977) coupled heparin to polymethylmethacrylate beads via its free amino group using carbodiimide activation. He found that heparinisation improved the compatibility of the material towards blood platelets, contrary to earlier reports by Salzman et al. (1969) and Merrill et al. (1970). Similar thromboresistance has been suggested for cynogen bromide bonded heparin (Hoffman et al., 1972) and acylated heparin (Plato et al., 1979).

Heparin can be covalently bound to any material that contains functional groups at its surface. One of the most common functional group in the substrate used to bind heparin is the hydroxyl group. Polyvinyl alcohol contains secondary hydroxyl groups that can be easily derivitised to bind heparin. Another potential material is polyethylene oxide or polyethylene glycol that contains two terminal hydroxyl groups on each molecule. The higher reactivity of the primary hydroxyl groups on the PEO compensates for the lower number of hydroxyls found on the molecule. Both these material form hydrophilic gels and are amorphous in the swollen state. They appear to have the basic requirements needed for a good blood contacting surface.

2.8 Polyvinyl Alcohol (PVA)

PVA has the following structure:

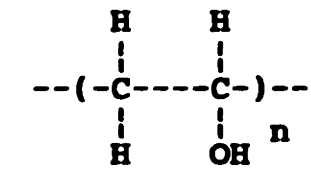


Figure 2-7: Stucture of PVA

Commercially, PVA is prepared by the alkaline hydrolysis of polyvinyl acetate. The degree of hydrolysis may vary, leading to samples with different degrees of acetylation. Even "fully hydrolysed" commercial PVA usually has about 1 mole% of residual acetate groups.

Vinyl acetate monomer polymerise mostly in a head to tail arrangement with about 1% going head to head, producing glycol structures in PVA.

head-tail head-head

Figure 2-8: Structure of PVA

The physical and chemical properties of PVA depend to a great extent on the method of preparation of the polymer and the degree of acetylation. Fully hydrolysed PVA has a glass transition temperature (T_g) of 85°C and a crystalline melting point range of 220°C to 240°C (Pritchard, 1970). X-ray diffraction detects the presence of poorly developed, randomly oriented crystalline structure of the fringed micelle type. It is usually only about 40% crystalline.

The solubility of PVA in cold water varies with the degree of hydrolysis. It increases with increasing percentage of hydrolysis up to about 88%, beyond which it decreases. Fully hydrolysed PVA is soluble in water at temperatures greater or equal to 90°C. Hot water (temp.>90°C) appears to be the only practical solvent for fully hydrolysed PVA. In aqueous solution, the PVA chain is more expanded as compared to its unperturbed state. The relatively large size of the PVA coils in water could be due to intra-molecular hydrogen bonding to form six-membered rings (Fujiwara et al., 1966). Intermolecular H-bonding is also usually present in aqueous solution of PVA.

Evidence from a study of phase transitions in aqueous PVA showed that PVA molecules are only completely separated at temperatures above 35°C and at concentrations below 0.25 g/dl. At lower temperatures, even at higher dilutions, the

molecules appear to be associated with each other due to hydrogen bonding between the hydroxyl groups (Soeya and Kono, 1957).

2.9 Chemical Crosslinking Of PVA

When a strong hydrogen bonding agent is added to an aqueous solution of PVA a thermoreversible gel is formed. PVA condenses with p-sulphonamidobenzaldehyde at 60°C in a mixed solvent of glacial acetic acid and phosphoric acid. A similar phenomenon is observed with epichlorohydrin at 50°C.

Polybasic inorganic acids like boric acid complexes with PVA in the same way as it does with sugars and other polyhydroxyl compounds (Lorand et al., 1959). The borate ion $(B_4O_7.10H_2O)^{2-}$, complexes more efficiently with PVA than boric acid (H_3BO_3), giving the corresponding polyanion (Figure 2-9). Table 2-2 (Finch, 1981) shows the behavior of fully hydrolysed PVA of different degree of polymerisation with borax ($Na_2B_4O_7.10H_2O$) and boric acid at 20°C and 60°C. Higher molecular weight PVA gels more easily. Lower temperature also favour the above cross-linking reaction. The reaction is almost instantaneous at room temperature (pH > 8.0).

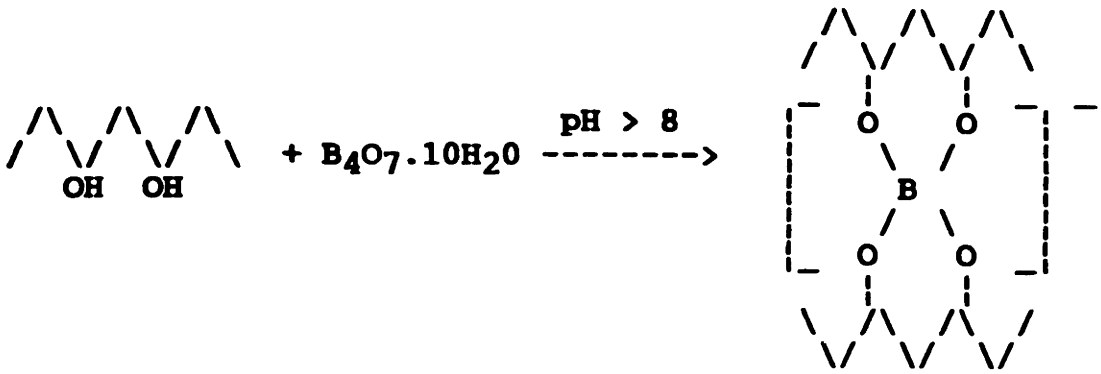


Figure 2-9: Reaction of PVA and Borate

The reaction can be easily reversed in a large excess of water.

Gel formation occurs in solutions containing 1 to 2% of borate and 2% or more of PVA. These gels have a definite melting range. Aqueous gel containing 2% borate and 5-10% PVA melts inthe range of 30-40^{o}C (Ushakov et al., 1961)

2.10 Radiation Cross-Linking of Polymers

Comprehensive reviews of the effect of ionising radiation on polymers are given by Chapiro (1962) and Bovey (1958). Most of the radiation studies have been done with gamma-rays or electron beams. Cobalt-60 is the most common source of gamma-rays while electron accelerators such as the Van de Graaff are used to deliver

Concentration of polyvinyl alcohol solution (% by wt.)		10		8		5		3	
Nominal degree of polymerization	Temperature (°C)	Boric acid	Borax	Boric acid	Borax	Boric acid	Borax	Boric acid	Borax
2010	20	2.7	0.1	3.1	0.2	4.0	0.5	4.0	1.2
2010	60	> 12	0.6	> 12	3.1	> 12	7.3	>12	18
1470	20	4.0	0.3	4.5	0.5	5.0	1.0	> 5	1.5
1470	60	> 12	4.6	> 12	7.6	> 12	15	> 12	> 20
980	20	4.5	0.8	4.5	0.9	5.0	1.2	5	1.7
980	60	> 12	1.3	> 12	14	> 12	18	> 12	> 20
300	20	5	1.0	5	1.3	1.5	1.5	5	2.0
300	60	> 12	1.5	> 12	> 20	> 12	20	> 12	> 20

Table 2-2: Effect of degree of polymerisation, concentration and temperature on the gelling of PVA by boric acid and borax (Finch, 1981).

electrons with energies up to 10 MeV.

The penetrating power of an electron beam is proportional to the energy of the beam. For the 3 MeV electron beam at the M.I.T. High Voltage Research Laboratory, a uniform penetration of the electron beam is obtained up to a depth of about 2.5 mm (Peppas, 1973).

Ionising rays can cause polymers to both degrade and cross-link. The relative amount of each determines whether the net effect on the polymer will be cross-linked or degraded. In general, it has been found that vinyl polymers with one or less substitution group will have net crosslinking, while those with two substitution groups will degrade(Figure 2-10), that is:

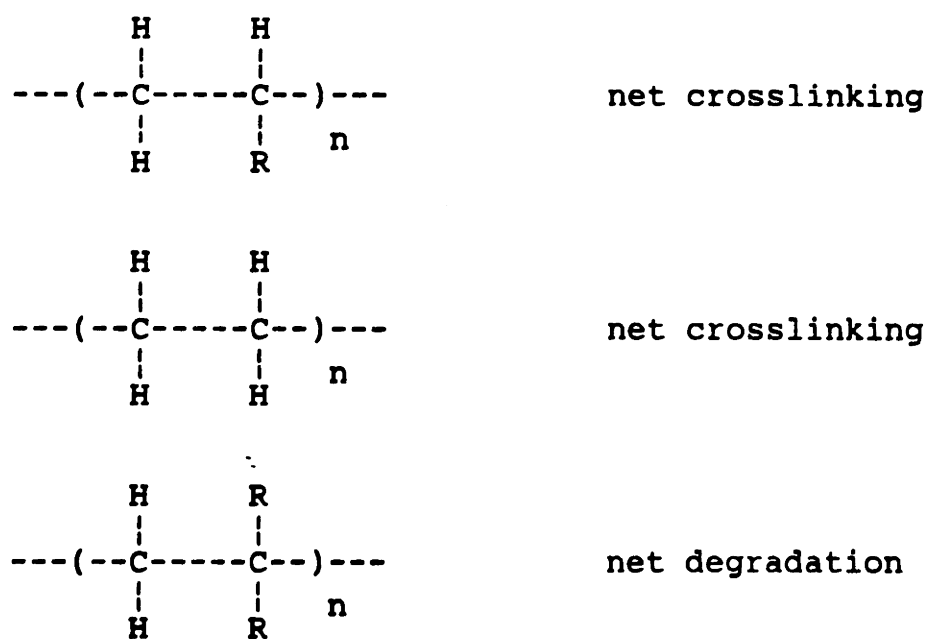


Figure 2-10: Polymer Structure that degrade or crosslink when irradiated

2.11 Effect Of Ionising Radiation On PVA

Crosslinking via irradiation is a radical process. A H· or OH· radical is abstracted from the main chain carbon either by the direct effect of the radiation beam in pure PVA melts/solid or by water radicals produced in the aqueous solution of PVA. Two macroradicals of PVA may combine to form a crosslink with a junction functionality of four. At the same time a competing process called scission can occur. The relative rates of scission over crosslinking depends on a number of factors such as the mobility of the macroradical, the concentration of the macroradical and the presence of diradicals such as oxygen and hydrogen peroxide. Since crosslinking involves the coupling of two macroradicals i.e. it is second order with respect to macroradical concentration, conditions that increases its mobility and concentration will favour crosslinking.

The mechanism through which scission occurs in not completely clear. Experimental evidences (Danno,1958; Abe,1959) imply that the scission is a first order reaction with respect to macroradical concentration and hence is not a strong function of temperature or macroradical mobility.

When solid PVA was irradiated at temperatures greater than its glass transition temperature (T_g =85°C), the net result was crosslinking (Sakurada et al.,1959). Above its T_g, two PVA macroradical are able to approach each other resulting in coupling. The minimum gelation dose is the dose at which a permanent gel or network is first formed. It is a good indicator of crosslinking efficiency. The minimum gelation dose decteases as the irradiation temperature increased from 90°C to 170°C (Abe,1959) due to its increased mobility at higher temperature.

Irradiation of solid PVA at room temperature results primarily in degradation (Danno, 1958) since the glass transition temperature of PVA is above room temperature. Analysis of the gas produced during irradiation yielded more than 91% of hydrogen. Infra-red analysis showed an increase in carbonyl groups consistent with both main chain rupture and intramolecular dehydration (Figures 2-11 and 2-12).

Figure 2-13 (Schnabel,1967) demonstrate the effect of concentration of polydimethylsiloxane in toluene on the efficiency of crosslinking and scission when irradiated with e- beam. Scission is practically independent of polymer concentration while crosslinking shows a very strong dependence on polymer concentration.

In aqueous solution, the PVA macroradical is more

mobile. Gelation will occur at lower PVA concentrations and radiation doses. The presence of water further increases the efficiency of crosslinking. Water radicals readily attack the PVA chains and hence increases the concentration of the

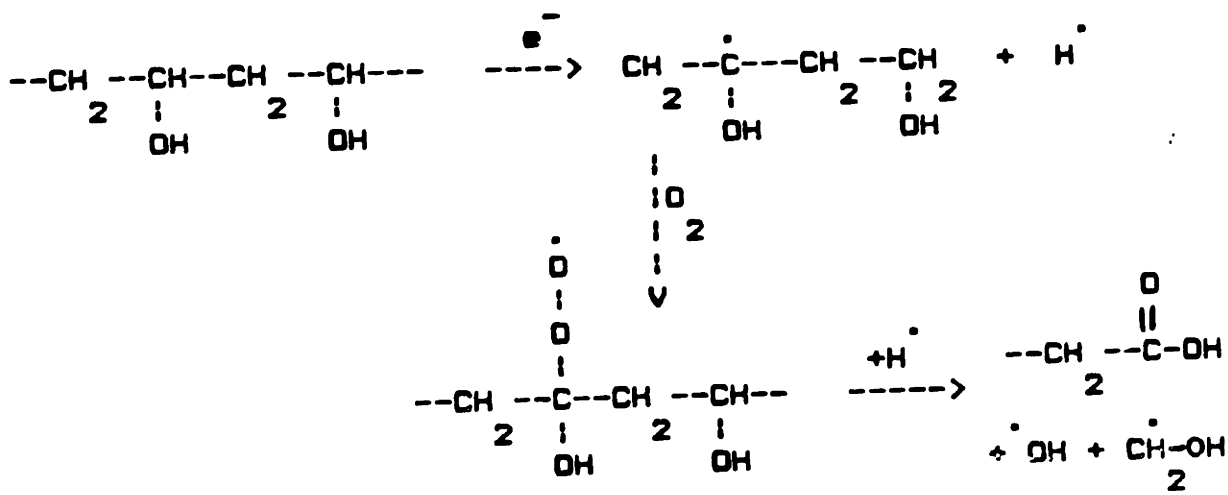
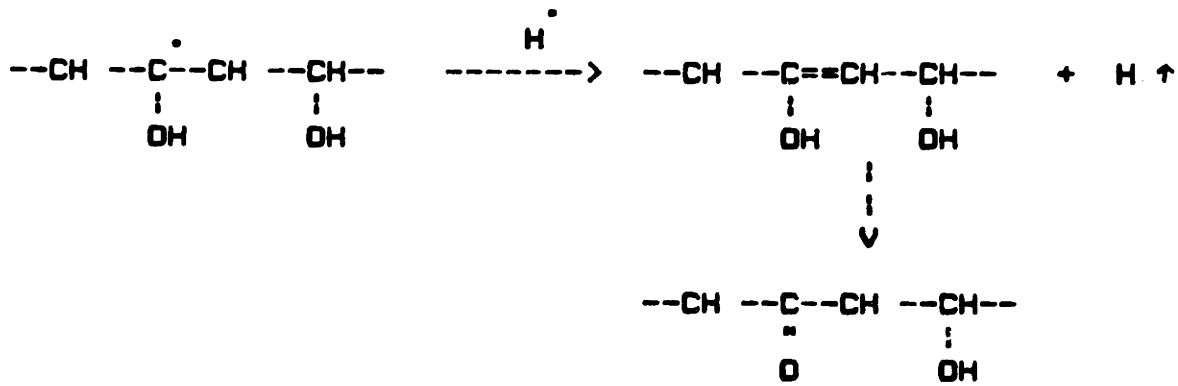


Figure 2-11: Main chain rupture

Figure 2-12: Intramolecular dehydration



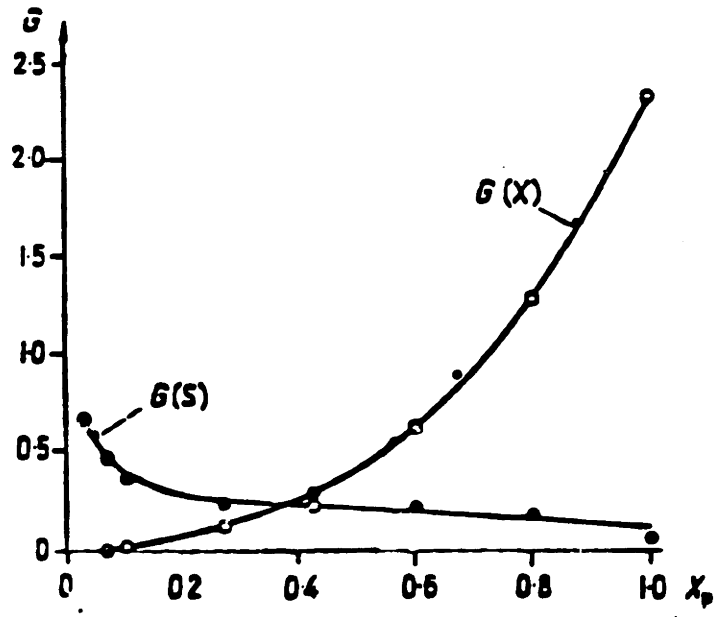


Figure 2-13: 100 eV yields of crosslinks [G(X)] and main chain scissions [G(S)] versus the mole fraction of poly-dimethyl siloxane in toluene

macroradical. Berkowitch et al. (1957) studied the effect of gamma-rays and 2 MeV electrons on aqueous solution of PVA (M.Wt. = 27,000 to 400,000) and noted that the critical concentration of PVA needed to form a macrogel is 0.32 - 0.67 g/dl. Below this concentration, intramolecular crosslinking predominates, forming microgels.

Danno (1958b) used fully hydrolysed PVA to develop an equation relating gelation dose, degree of polymerisation and concentration of PVA:

$$(D_g \times P) = (5.1 \times 10^8) + (1.37 \times 10^8 \times C)$$

where: D_g --- gelation dose

P --- degree of polymerisation

C --- concentration in g/dl

Allowing the PVA solution to stand before irradiation has been shown to increase crosslinking, evidently due to the PVA coils adopting some form of preferred conformation (Sakurada and Ikada, 1961).

2.12 Radiation In Aqueous Solution

Irradiation of water with high energy beams will set off the following reactions:

$$H_2O \text{ --------> } e^- + H_2O^{\cdot +} + H_2O^* \quad (H_2O^*: \text{activated water})$$

$$e^- + nH_2O \text{ -----> } e^-_{aq} \quad (\text{hydrated electron})$$

$$H_2O^+ \text{ --------> } H^+ + OH\cdot$$

$$H_2O^* \text{ -------> } H\cdot + OH\cdot$$

$$H_2O^* \text{ -------> } e^- + H_2O^+$$

Figure 2-14: Primary Products of Irradiated water

Some of these products can recombine to form hydrogen gas(H_2), water (H_2O) or hydrogen peroxide (H_2O_2). Others will attack the PVA to form PVA macroradical which then can undergo scission or crosslink.

$$H\cdot + \cdot OH \text{ ------> } H_2O$$

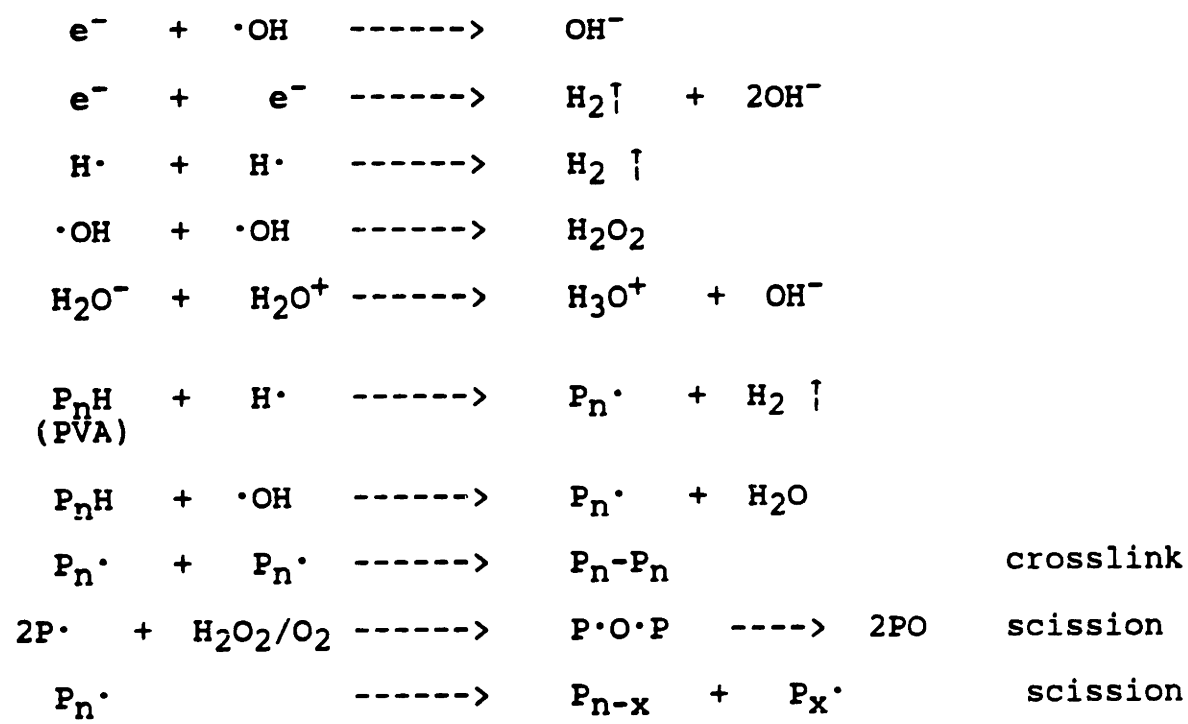


Figure 2-15: Reactions involved in the irradiation crosslink of PVA

The scission product is generally a carbonyl. The presence of O_2 and H_2O_2 could concievably increase the rate of scission. Oxygen acts as an unstable diradical and may get incorporated in the chain to form an unstable crosslink which then degrades easily. Therefore, irradiation is uaually carried out in an inert atmosphere.

Systematic studies on the irradiation of PVA solution with the 3MeV electron beam have been carried out at MIT (Bray,1972; Peppas,1973). These authors found that for a given initial concentration and molecular weight, the number

average molecular weight between two topological junction (M_c) decreased with increasing dose up to a limiting value beyond which M_c remains constant or may even increase. Irradiation of the PVA solution under oxygen did not affect the M_c values which implies that the mechanism whereby the oxygen incorporated into the chain and hence leading to scission is not important under the condition used. The dose rate delivered under the electron beam appeared to be high enough to consume the oxygen in the solution.

The hydrogels produced by Bray(1972) were transparent but marred by numerous air bubbles in the gel. The amount of air bubbles in the gel decreased as the concentration of the PVA increased. This could be due to a number of reasons. The higher concentration of polymer chains may be able to trap the H· radical more effectively thus decreasing the amount of H_2 gas formed by recombination of two H·radicals; H_2 being the main gas produced during irradiation (Danno,1958). The viscosity of PVA solution increases rapidly with concentration. A higher partial pressure of H_2 would be needed to form gas bubbles in the more viscous solution. This would also lower the number of gas bubbles formed.

PEO hydrogels were made under similar conditions and contains an order of magnitude more bubbles than PVA hydrogels (Dennison,1986). PEO solutions of comparable

molecular weights and concentration were less viscous than the corresponding PVA solution. The air bubbles in the PVA solution were almost completely removed by freezing the PVA solution at -20°C and thawing it at room temperature before irradiation (Peppas, 1973). The viscosity of the resulting solution is very high - a 5% solution (M_v ≈108,000) after undergoing the above freeze thaw treatment is almost a solid gel. Both the solutions and the resulting gels were cloudy due to the formation of "supramolecule" or microcrystalline regions. Preliminary results done in our laboratory showed that these supramolecular regions in the gels are permanent and could not be removed by heating. This indicate that crosslinking occured in these regions "fixing" the microcrystalline structures permanently in the gel.

2.13 Immobilisation Techniques

Techniques for immobilising cells and enzymes to substrates have been used for many years by biochemists. These techniques ranged from physical adsorption, ionic bonding, entrapment to covalent bonding of the ligand to the substrates. For long term application of the immobilised species, covalent bonding is the only suitable method of immobilisation.

2.13.1 Covalent Bonding Methods

The functional groups on the biological molecules that usually take part in the covalent bond are amino, carboxylic, sulfhydryl, hydroxyls, imidazole and phenolic groups. During coupling these functional groups react with the insoluble substrate or carrier containing reactive moieties such as diazonium, acid azide, isocyanates, halides or carbonyl imidazoles.

The substrates are usually inert and have to be derivitised to contain the active moieties above. A variety of methods have been developed to activate hydroxyl groups. Cynogen bromide is by far the most common technique used due to its ease of use and high reactivity. Another advantage of using CNBr is that activation can be carried out in aqueous medium.

The chemical reactions of CNBr are complex; cyanate esters, cyclic imidocarbonate, linear imidocarbonate or carbamate can be formed during the activation. The cyanate ester is very reactive while the carbamate is totally inert (Wilchek et al., 1984). The major disadvantages of using this reagent are its high toxicity, the partial charge on the isourea bond formed which caused nonspecific adsorption onto the substrate. The isourea bond is also unstable and the ligands will leach slowly from the substrate.

Other coupling reagents were used to replace CNBr. Epoxy activation using bisoxirane or 1,3-bytanediol couples with primary amines as well as hydroxyls forming stable bond. Elimination of excess unreacted oxirane function is however difficult requiring either prolonged storage in alkaline solution or prolonged treatment with small nucleophiles (Sundberg and Porath,1974). Similar problems exist with divinylsulphone.

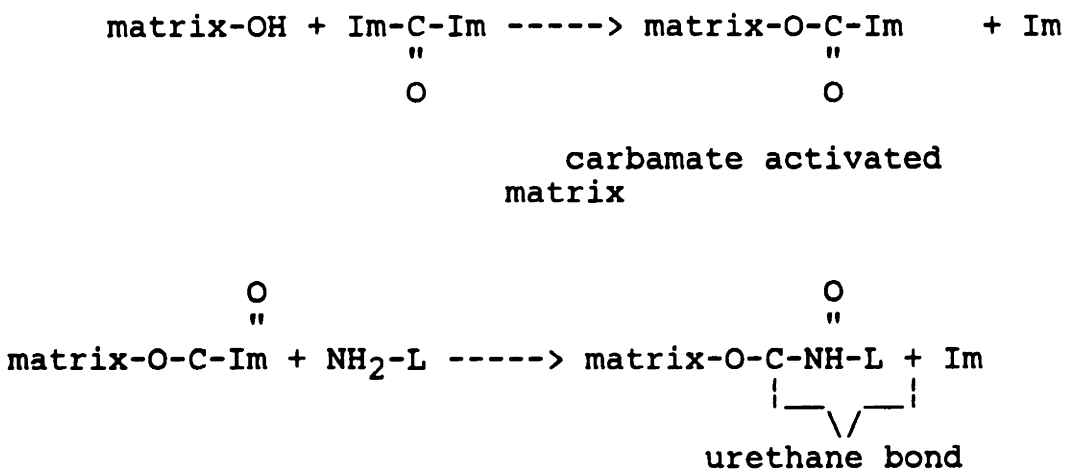
Two promising reagents that have been used in immobilising enzymes are carbonyl diimidazole and sulphonyl chlorides. Both of these have reactivities comparable to that of cynogen bromide without its problems of partial charges and instability. They are however sensitive to hydrolysis by water and hence activation is carried out in dry polar organic solvents. These two reagents are reviewed more extensively below.

2.13.2 Carbonyl Diimidazole

Carbonyldiimidazole (CDI) was first introduced as an activating agent for hydroxyl-containing matrix by Bethell et al. (1979). The carbonylated matrices, when coupled with monoalkylamines, were found to be devoid of any additional charged group (Bethell et al., 1981a).

The imidazoyl carbamate group (see Figure 2-16) formed

was found to be susceptible to nucleophilic attack by amines in aqueous or organic solvent but is relatively stable towards oxygen nucleophiles (e.g. water).



where: Im = imidazole

L = ligand.

Figure 2-16: Coupling by CDI activation

At pH 9.0 and pH 10.0, the total time to hydrolyse all active groups on activated sepharose was found to be 30 hours and 10 hours respectively (Hearn et al., 1979). This enables coupling of ligand to be done in an aqueous media.

The active carbamates couple smoothly with ligands containing free amino groups to give a non-basic urethane (N-alkyl carbamate) derivative. Hearn et al. (1979) successfully bound a number of different aminoligands with different pKa's, i.e. proteins (e.g. trypsin, BSA, human thyroglobulin), 6-aminohexanoic acid (pKa=10) and glycine (pKa=9.8), to the carbamate activated matrices. The immobilised proteins have been demonstrated to be able to separate other proteins in affinity chromatography. They were able to obtain good coupling yields in the pH range of 8 to 10, provided that the buffer capacity was sufficiently high to prevent significant pH changes. The yields were highest when the coupling was done at a pH of at least one unit above the pKa value of the amine group on the ligand (Bethell et al., 1981b).

This method of binding proteins to a support compares very favorably with the cynogen bormide (CNBr) method. The latter produced only 2% activation yield (Cuatrecasas, 1970) based on CNBr reagent, while CDI was reported to give yields of as high as 40-45% based on CDI reagent (Hearn et al., 1979).

The activated matrices were stable when stored in dry acetone at 4^{o}C for as long as 6 months. At room temperature in dioxane, the percentage of activated groups remaining was 60% of the original after 14 days (Bethell et al., 1981a).

In the same study, the researchers showed that at pH 7, the % loss of the coupled ligand (in this case, glycine) was about only about 0.3% of the total amount of glycine bound after 42 days and this did not increase significantly with the time of incubation.

Any unreacted carbamate groups on the matrix will be hydrolysed by water during or after the coupling stage, regenarating the hydroxyl groups on the matrix. This eliminates the additional step of blocking the unreacted carbamate groups. This method also does not introduce new groups onto the matrix.

From these series of studies, it appears that immobilization of amine-containing ligand could be most conveniently prepared by CDI activation.

2.13.3 Sulphonyl Chlorides

Arenesulphonate groups have been known to be exellent leaving groups since the introduction of the Tipson procedure for the synthesis of p-tolunesulphonates(tosylates) (Coates and Chen,1969). Truce and Norell (1963) patented the first method for the synthesis of 2,2,2-trifuluroethanesulphonyl

chloride (Tresyl chloride) with a reactivity 100 times that of the tosylates (Crossland et al.,1971). The yield obtained by their method is however very low. Tresyl chloride became commercially availabe only when a more efficient route of synthesis was developed by Crossland and coworkers(1970) giving 41% yield.

Tosylated agarose has been successfully used for the immobilisation of affinity ligands and proteins such as trypsin inhibitors and alcohol dehydrogenase at pH 9.7 and 8.5 respectively (Nilsson and Mosbach,1980). The amount of activated groups present was monitored through the UV absorption of the toluene groups. The ligand are bound directly to the carbon atom of the support and no known side reaction have been detected during the activation as well as the coupling process. The large hydrophopic toluene groups can in some instances change the thermodynamic interaction between the support and the solvent causing a decrease in the swelling of the support. This will greatly decrease the availability of the activated group to coupling and also increase the difficulty of removing the excess unreacted groups.

Tresyl chloride was later introduced as a coupling reagent (Nilsson and Mosbach, 1981). Its leaving properties is superior to those of tosyl chloride (Crossland et al.;1971; Nilsson and Mosbach,1984) with a less bulky side

group. Coupling of the ligands and tresylated supports is facile and requires mild conditions. The pH range at which significant amount of ligands are bound is between 7.5 to 9.5. Functional groups on the ligand that can attack tresyl chloride activated matrices include thiols, amines, imidazoles and phenolics.

The activated matrices of both the tosylates and tresylates are stable in 3mM aqueous HCl at 4°C for at least 3 months (Nilsson and Mosbach,1981) in contrast to CNBr activated matrices which have a half-life of about 30 minutes (Kohn and Wilchek, 1978). The mechanism of activation of the sulphonyl chlorides are as follows:

ACTIVATION

$$R\text{-}CH_2\text{-}OH + Cl\text{-}SO_2\text{-}CH_2CF_3 \xrightarrow{\text{PYRIDINE}} R\text{-}CH_2\text{-}OSO_2\text{-}CH_2CF_3$$

TRESYL CHLORIDE

$$R\text{-}CH_2\text{-}OH + Cl\text{-}SO_2\text{-}C_6H_4CH_3 \xrightarrow{\text{PYRIDINE}} R\text{-}CH_2\text{-}OSO_2\text{-}CH_2CF_3$$

TOSYL CHLORIDE

COUPLING

$R\text{-}CH_2\text{-}OSO_2\text{-}X + H_2N\text{-}L \text{ -------> } R\text{-}CH_2\text{-}NH\text{-}L + HOSO_2X$

$R\text{-}CH_2\text{-}SO_2\text{-}X + SH\text{-}L \text{ -------> } R\text{-}CH_2\text{-}S\text{-}L + HOSO_2X$

where

X = CH_2CF_3 OR $C_6H_4CH_3$

L = LIGAND

A modified form of tresyl chloride has been synthesized whereby a chromophore is attached to the molecule (Scouten and van Der Tweel,1984). The authors were able to visually follow the activation and coupling of ligands to various substrate using the chromophoric tresyl chloride. They found that coupling to primary suphonates is facile but that of secondary sulphonates occur only with difficulty. This is almost certainly to be true for all types of coupling reagents. For matrices with both primary and secondary hydroxyl groups, performing the activation at low reagent concentration and temperature will limit the derivitisation to only primary hydroxyl groups.

3. OBJECTIVE

The objective of this thesis is to study the factors affecting the thrombin inhibiting activity of immobilised heparin to hydrogels.

3.1 Specific Aims

1. Synthesis and characterisation of PVA hydrogel network without the introduction of other chemical groups by radiation crosslinking of PVA in aqueous solution.

2. Effect of temperature on the amount of heparin bound and the activity of the immobilised heparin as determined by thrombin inhibition.

3. To study the effect of the following factors on the thrombin inhibiting activity of immobilised heparin :

   a. the degree of activation of the PVA hydrogel
   b. the terminal amine on the heparin
   c. the length of the leash to which heparin is connected to the network

3.2 Assumptions and Experimental system

3.2.1 Heparin

Commercially purified heparin was assumed to be pure and free from other polysaccharides. Diosynth heparin from the Chicago plant was used since this heparin had undergone only a mild oxidation process. Preliminary study showed that this heparin has one of the highest serine content amoung the commercial heparin.

The heparin however is heterogenous in terms of its chemical composition, biological activity and molecular weight. Unfortunately the molecule is too complex to completely characterised it in this study; hence whole heparin was used throughout this thesis without any further purification or modification except when noted.

Thrombin which cleaves fibrinogen to fibrin can be produced via the intrinsic and extrinsic clotting mechanism. For this study the biological activity of heparin was measured in terms of its ability to inhibit the hydrolytic activity of thrombin. It is assumed that bound heparin that can inhibit thrombin will increase the biocompatibility of the material.

3.2.2 Hydrogel

The surface of hydrogels by its very nature is diffuse and undefined as schematised in figure 3-1. The polymer content of the hydrogels in all instances is less than 10%.

Therefore "surface" of hydrogel may extend up to 20 - 30 angstroms with strands of polymer molecules moving freely in it. Based on this model alone it is to be expected that some of the heparin that is bound to the surface may not be able to deactivate thrombin even though it may retain its activity strictly due to steric hindrances.

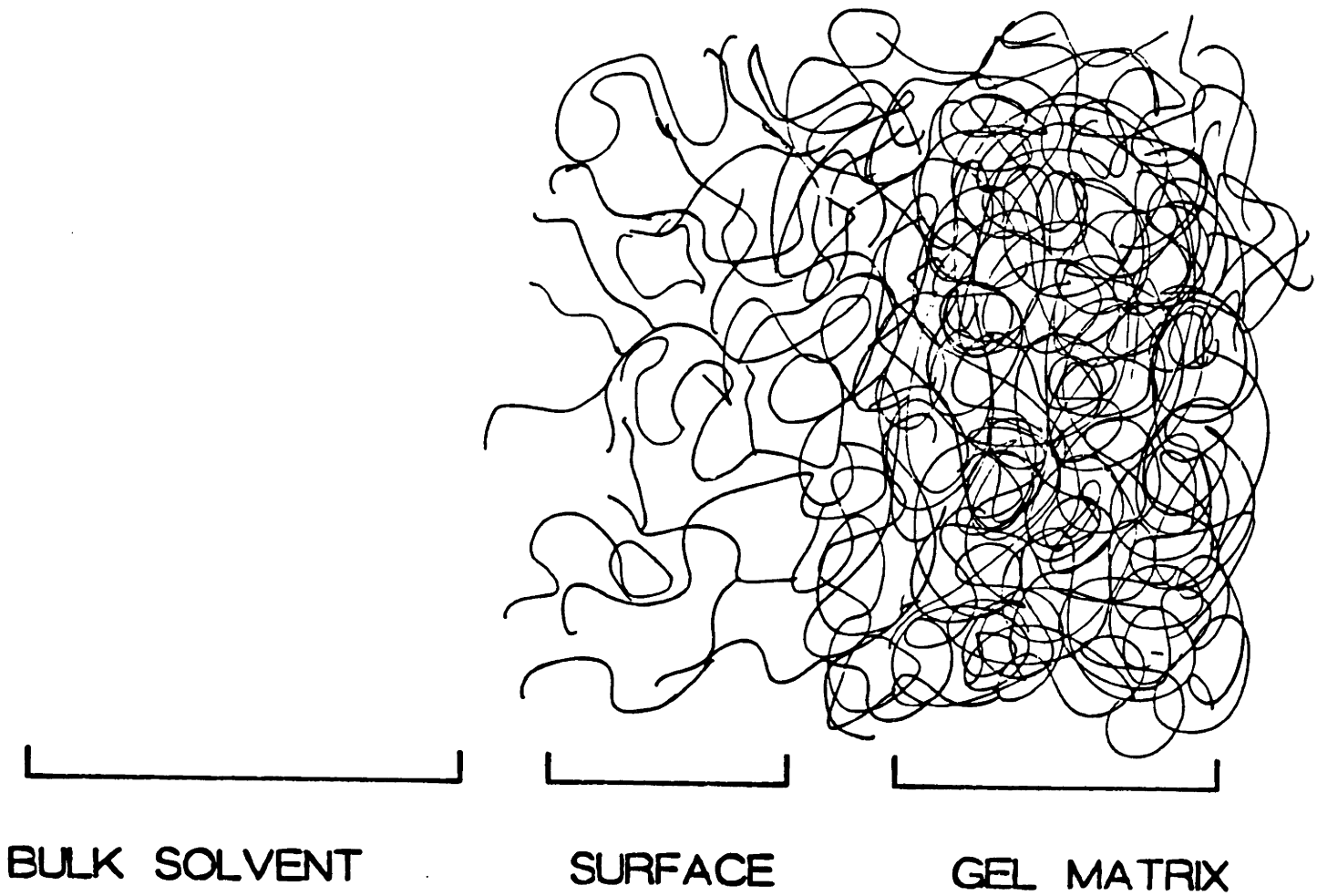


Figure 3-1 : Schematic Diagram of the Surface of Hydrogels

4. MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Polyvinyl alcohol

Polyvinyl alcohol was purchased from PolySciences, Inc., Warrington, PA. It is 99% hydrolysed with a viscosity average molecular weight of 108,000.

4.1.2 Polyethylene oxide

Polyethylene oxide was purchased from Fluka AG, Switzerland. It is monodisperse with a molecular weight distribution (M_w/M_n) of about 1.02 and a molecular weight of 35000. Each of the terminal residues of the molecule carries a hydroxyl group.

4.1.3 Heparin

Porcine mucosa sodium heparinate was kindly donated by Diosynth, Inc., Chicage, IL. The purified heparin (lot #052782) has a specific activity of 158 U/mg, while the crude heparin (lot #LC011684AB) has a specific activity of 128.1 U/mg. Crude heparin is obtained at the stage immediately after extraction from porcine mucosa without undergoing the oxidation and purification steps. Low

molecular heparin was be prepared by nitrous acid degradation at high pH and aminated at the reducing ends.

Heparin standards used were prepared by Drs Lormeau and Petitou at Choay Chimie, France by partial nitrous acid degradation and fractionation on size exclusion columns. The molecular weights of these fractions were independently determined by Low Angle Laser Light Scattering detector together with a regular refractive index detector (supplied by Dr. Gil van Dedem of Diosynth BV, Oss, Netherlands).

4.1.4 Chemicals

Carbonyldiimidazole, cyanoborohydride, hexadiamine, Dowex 20 cation exchanger and the globular proteins was obtained from Sigma Chemical Co., Mo. The scintillation cocktail, hydrofluor, was from National Diagnostic, NJ. 2,2,2-trifloiroethanesulphonyl chloride was purchased in 1ml sealed ampoules from Fluka A.G.. After an ampoule was broken the contents were stored in a dry glass vial and kept dessicated at all times in the refrigerator. Tresyl chloride that was more than 2 months old was discarded.

H.D.-phenylalanyl-Lpopecolyl-l-arginine-p-nitroanilide dohydrochloride(S2238) was obtained from KabiVitrum through Helena Labs, Texas. Electrophoresis reagents were obtained from Biorad. All other solvents and reagents used were at least of reagent grades. Solvents that were used in the activation of the hydrogels were dried over 4 A molecular

sieves before use.

4.1.4 Labelled Material

^{35}S-heparin, ^{14}C-serine, ^{14}C-glycerol were purchased from Amersham Co.

4.1.5 Antithrombin and Thrombin

Antithrombin was purified from outdated blood plasma obtained from the American Red Cross Society,Wash. D.C. with the assistance of Dr. J. Lindon of the Beth Israel Hospital by affinity chromatography using the method developed by Prof. Rosenberg research group at MIT.

Purified thrombin was kindly donated by Dr. John Fenton of the New York State Dept of Health at Albany, NY. Both these proteins were run on an SDS electrophoresis gel to check for purity and activity. The concentration of antithrombin was determined using UV spectroscopy and stored at -70°C or in 50% glycerol - PBS solution at -20°C.

4.1.6 Plasma

The plasma used in this study was platelet rich plasma prepared from fresh blood by Dr. J. Lindon of the Beth Isreal Hospital. Citrated whole human blood was spun at about 1600 g for 2 minutes to remove the cells from the blood. This preparation has 100,000 to 600,000

platelets per ml plasma. The plasma was used immediately after preparation.

4.1.7 Radiation Facility

A van de Graaf accelerator located at the High Voltage Research Laboratory at MIT was operated by Mr. Kenneth Wright. The accelerator delivered 3 MeV electrons at a beam current of 160 uAmp. With the appropriate overlay placed over the samples the beam penetrating into the sample was essentially monoenergetic with about 1% spread in energy.

Radiation dose rate delivered to the sample was controlled by the speed of the sample conveyor belt that ran beneath the window and the beam current. The complete setup of the facility was documented by Peppas(1973). Figure 4.1 is a schematic diagram of the radiation facility setup used.

4.2 METHODS

4.2.1 Polyvinyl Alcohol solution

PVA solutions were prepared by adding a known weight of PVA crystals into deaerated ultrapure water containing 0.01% sodium azide (antibacterial agent) in a glass stoppered volumetric flask. The mixture was then transfered into a

glass stoppered conical flask, degassed by bubbling pure argon gas into the mixture for 15 minutes, and placed in a convectional oven at 90°C for 5 hours. The contents of the flask were gently swirled (to avoid foaming) to mix the

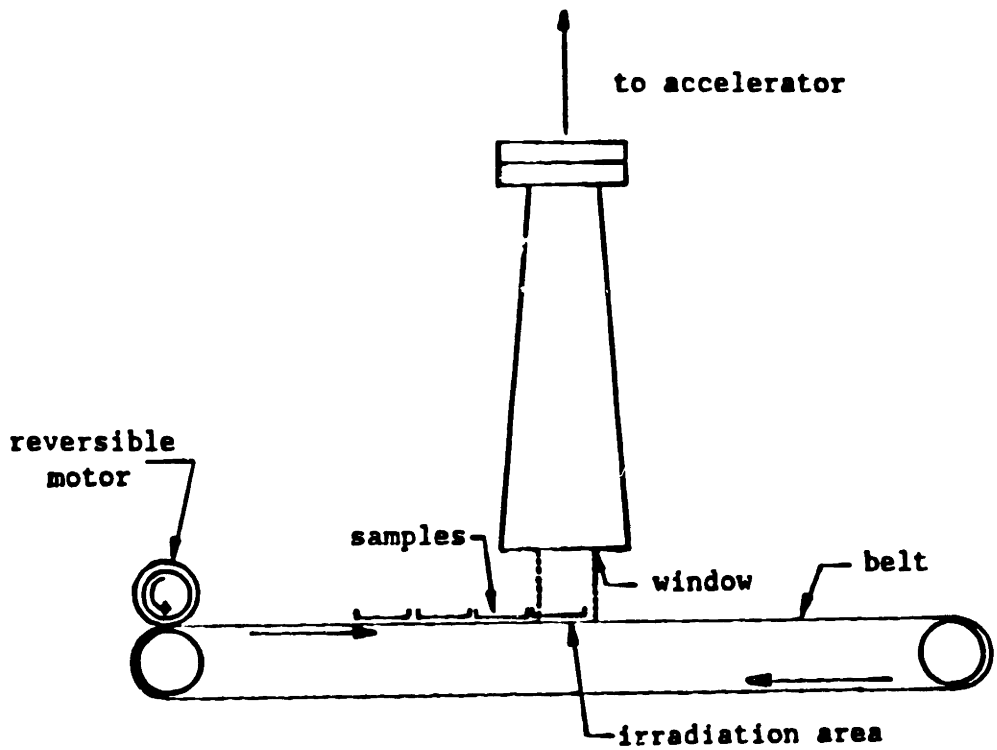


Figure 4.1 :Schematic Diagram of the Radiation Setup at the High Voltage Laboratory at MIT

solution after all the PVA has gone into solution. The swirling was repeated at about half hour interval until there was no apparent polymer concentration gradient in the solution as observed by the lines of polymer concentration interfaces on mixing.

4.2.2.1 PVA Gels

The hot PVA solution was quickly transfered to a glove box. The solution was kept at 80°C by incubating it in a water bath kept at that temperature in the glove box. The box was closed off and purged with argon gas for 20 minutes. The PVA solution was then transfered into polystyrene petridishes (Falcon) 60 X15 mm, with a 10cc disposable polypropylene syringe. The volume in each dish was calculated such that the depth of the solution was never more than 2.5mm. Uniform crosslinking throughout the gel was obtained only up to this depth (Peppas, 1973). The lower dish was wrapped with Saran-wrap held in place by a rubber band and the cover dish placed over it. The whole assembly was then

sealed in a heat sealable polyethylene bag to keep the solution under argon.

The sealed dishes were then removed from the glove box, after cooling to room temperature and immediately brought to the HVRL to be irradiated. The dishes were placed in a trough of cold water (17^{o}C) on the conveyor belt and subjected to multipasses under the electron beam of the van de Graaf generator until the desired dose was reached. The dose rate used for all the experiments was 150,000rad/sec.

4.2.2.2 PEO hydrogels

The PEO hydrogels were made in a similar manner as PVA by Kathleen Dennison (1986) at MIT. A 5 % solution of PEO in 0.5M potassium sulphate solution was irradiated with the e-beam as in the PVA hydrogel. The gel was extracted with water and then dried in the vacuum oven at 50^{o}C for 48 hours or till no more change in weight was observed. The PEO hydrogel was then left to swell in THF for 3 days with frequent changes in solvent to remove traces of water that might be in the gel.

4.2.3 Measurements of Mc

The gel was weighed immediately after removal from the petridish. The gel was then allowed to swell in fresh water, preserved with sodium azide, at 90^{o}C for 24 hours. This served the purpose of releasing any crystalline structure

that might have form and extraction of sol fractions in the gel. The extract was concentrated to a known volume and ran through the size exclusion chromatography (LC/GPC 150, Waters Assoc.) to determine the amount of extractables. The extractables were found to be less than 0.5% in all cases and was henceforth neglected in all the calculations. The gel was then placed in fresh water and allowed to swell to equilibrium at room temperature for seven days or until a constant weight was obtained. The swollen gel was weighed and then allowed to dry slowly in a vacuum oven at 60°C for 72 hours or till there was no more weight change.

The volume of the polymer fraction in the samples were calculated using a density of 1.269 g/cm^3 (Sakurada et al., 1959) for PVA as follows:

$$Vp = W_p/d_p$$

where V_p = volume of dried polymer

W_p = weight of dried polymer

d_p = density of polymer

The total volume of the sample was calculated by the simple addition of the volume of polymer and the volume of water in the sample taking the density of water to be 1 gm/cm^3.

The polymer volume fraction in the gel after radiation and at equilibrium swelling was calculated as follows:

$$V_{2,r} = \frac{V_{p,r}}{V_{p,r} + V_{w,r}}$$

$$V_{2,s} = \frac{V_{p,s}}{V_{p,s} + V_{w,s}}$$

where

V = volume

p = polymer

w = water

r = relaxed state i.e. just after irradiation

s = swollen state at equilibrium

$V_{2,r}$ = polymer fraction at the relaxed state

$V_{2,s}$ = polymer fraction at the swollen state

M_c was calculated from Bray's (1972) modification of Flory's affine deformation equation on equilibrium swelling of network(appendix A):

$$\frac{1}{M_c} = \frac{2}{M_n} - \left[\frac{V/V_1(\ln[1-V_{2s}] + V_{2s} + \chi_1 V_{2s}^2)}{V_{2r}([V_{2s}/V_{2r}]^{1/3} - 0.5[V_{2s}/V_{2r}])} \right]$$

V_{2r} = volume fraction of polymer immediately after irradiation.

V_{2s} = volume fraction of polymer at equilibrium swelling.

χ_1 = Flory's interaction factor for PVA (0.49, Sakurada et al.,1959)

V = specific volume of PVA

V_1 = molar volume of water

$\bar{M}_n$ = initial number average molecular weight of PVA.

Mn of the initial PVA molecule was determined by size exclusion chromatography on the Waters 150 LC/GPC instrument using narrow molecular weight distribution polyethylene oxide standards at 30°C.

4.2.4 Molecular weight determination

The molecular weights of PVA and heparin samples were analysed on the LC/GPC 150 (Waters Associates) size exclusion chromatography. Three SEC columns were used in series viz. Toyo Soda TSK G5000PW, TSK G4000PW and TSK G30000PW. The data from the SEC was collected and analysed on the IBM using a program developed by K.A. Dennison (1986).

The mobile phase for PVA was 0.01% azide solution in ultrapure water from the milli-Q water purification system

(Millipore Corp.). Since no PVA molecular weight standards were available narrow molecular weight PEO were used to calibrate the columns. A universal calibration curve of log [M][N] of PEO versus retention time was plotted and from there the PVA "standards" were calculated; where

$$[\eta] = KM^{\alpha}$$

$$\log [M][N] = \log KM^{1+\alpha}$$

The values K and α used for PVA were (Brandrup and Immergut, 1975) :

$$K = 45.3 \times 10^{-3}$$

$$\alpha = 0.64$$

and that for PEO were:

$$K = 12.5 \times 10^{-3}$$

$$\alpha = 0.78$$

All runs were carried out at $30^{o}C$

The mobile phase for heparin during the SEC were 0.05M, 0.1M and 0.15M NaCl with 0.01% NaN_3 solution in ultrapure water. The columns in these cases were then calibrated using the heparin standards from Choay Chimie. The standards are in the molecular weight range of 6000 -17000 daltons.

4.2.5 Protein Penetration Test

The method of Bauer(1972) was used. The gels to be tested were allowed to swell in the buffers in which the proteins were dissolved in (phosphate buffer saline in this case) for 48 hours. They were placed in 1% solution of the protein to be tested at room temperature for 24 hours. The samples were then transfered into a 0.33% Buffalo Black dye in 7.3% aqueous acetic acid solution for two hours. The gels were then destained in 7.3% acetic acid solution till no more dye was removed and the control gel (without the protein treatment) has turned colourless.

The protein molecules that have penetrated the gel matrix are fixed in there as well as stained when placed in the dye as they denature. Denaturation caused the protein to unfold and hence increased their effective diameter of diffusion. Their absorptivity onto the matrix may increase too. This prevents the protein in the gel from diffusing out during the washing stages which can take as long as a week. Gels which protein penetrated retained the blue color. This test gave a qualitative estimate of the ability of the gel to exclude proteins from its matrix since the effective diameter of the proteins used is known (appendix B).

4.2.6 Heparin Penetration

The hydrogel was equilibrated in the coupling buffer for 48 hours. Exact volumes of a 5% heparin solution in the coupling buffer was placed in glass vials. Pieces of gel

whose weight and polymer volume fractions were determined ahead of time were placed into each vial. The volume of gel placed into each vial was such that its solvent volume fraction was of the same magnitude as the heparin solution in the vial; i.e.gel whose total volume of water is 1.25ml in 1.5ml of heparin solution.

The vials were gently shaken at room temperature on a rocking table. At intervals between 5 minutes and 5 days the concentration of the heparin in the supernatant was determined using the carbazole assay for uronic acid of Bitter and Muir (1962) and a mass balance was performed on each vial. A decrease in heparin concentration in the supernatant was taken as an indication that heparin has penetrated into the matrix.

The same procedure was repeated with PVA gels that were first placed in 0.1M borate buffer at pH 8.5 for 5 minutes before adding the heparin solution. The presence of borate ions in the solution has been found in a preliminary experiment, not to affect the carbazole assay of heparin

4.2.7 Heparin Determination

The amount of heparin present in a solution was assayed by determining its uronic acid content via the carbazole reaction using the modified method of Bitter and Muri (1962). The uronic acid content was converted to heparin by contructing a standard curve from known heparin concentration. Heparin dissolved in a number of salt solutions were tested for the effect of the various salts on this assay. This assay was not affected by the presence of carbonate, sulphates or borate. Water used for making heparin solutions that were to be assayed by this method was preserved with sodium benzoate instead of azide as the latter inhibits the carbazole colour formation. Chlorides were also avoided as the colour produced was about 33% lower than in its absence.

Heparin was also determined by the modified method of Jacques and Wollin (1967) using Azure A dye.

Heparin bound to PVA hydrogel was assayed by the sulphur content of the substrate. Sulphur was analysed by an independent laboratory (Gailbraith Laboratory, Tennessee) using the Leco Sulphur analyser.

Heparin bound to PEO were analysed in the amino acid analyser for the glucoseamine content since PEO can be quantitatively hydrolysed in concentrated HCL. The hydrogel was hydrolysed in 5.7M HCl solution for 24 hours and the supernatant run on the Dionex 500 amino acid analyser and

eluted through the high pressure ion exchange column (Durum DC4A 8um beads). The glucoseamine was detected using ninhydrin reaction.

^{35}S-heparin was determined using the liquid scintillation counter (Beckman LS 120). For ^{35}S-heparin bound to the hydrogel, the scintillation cocktail was mixed with 20% water to form a gel in which the hydrogel particles was suspended.

4.2.8 Fluorescamine Assay of Free Amino Groups

Free amino groups was assayed using the method of Udenfriend and coworkers (1972). Fluorescamine was reacted with the primary amines present in the sample at pH of 9. The fluorescence was recorded on the Aminco-Bowman Fluorospectrophotometer (American Instruments Co.). The cuvette chamber was water jacketed and modified such that a magnetic stirrer can be placed beneath it. The sample was preincubated in a precision water bath set at 37^{o}C and the water circulated through the cuvette chamber. This was done to minimize the drift in the fluorescent reading due to temperature differences between the sample and the chamber. The samples were constantly stirred during the reading to ensure uniformity in temperature throughout the sample.

The excitation wavelength used was 390nm and the emmission wavelength 475nm. The slit width of both the excitation and emmission side was set at 5nm.

4.2.9 Determination of the Carboxyl Groups

An aliquot of a heparin solution was passed through a column of strongly acidic cation exchanger (Dowex 50), 7 inches in length, 5/8 inch in diameter, at a flow rate of 8 drops per minute. The eluent from the column was collected until the eluent from the column was neutral. The eluent was then titrated with a standard 0.01M NaOH solution and the pH read off the pH meter (Corning, 125). The amount of carboxyl groups present was calculated from the amount of NaOH used in going from pH 4.35 to 8.5. The column was regenarated by passing 3M HCl through the column. A sample of the titration curve obtained is given in Appendix C.

4.2.10 Amino Acid Analysis

The sample solution was first desalted by passing it through a sephadex G-25 prepacked column (Pharmacia Chemicals, PD-10). The desalted eluent was then freeze dried followed by hydrolysis in redistilled HCl (5.7M) for 24 hours. The hydrolysate was analysed on the HPLC amino acid analyser (Dionex 500). The column was an ion exchange column (Durum DC 4A, 8um beads) and the sample eluted with buffers at pHs 2.2, 3.25, 4.25 and 7.9 sequentially. The amino acid eluting out was reacted with ninhydrin and the detected with a uv/vis detector at 590nm and 440nm. Standard solutions of

amino acids including serine and glucoseamine were used to calibrate the columns.

4.2.11 Reducing Group Determination

The number of reducing terminals found in a solution of heparin was determined using the Shaffer-Somogyi micro methods for reducing sugars. The reducing terminals were oxidised by excess cupric ion to cuprous ion. Unreduced cupric ion was reduced by iodide ion which in turn get oxidised to free iodine molecule. The amount of iodine released was then assayed by titration with standard sodium thiosulphate solution. The difference between the volume of thiosulphate solution required to neutralise iodine in the sample and the blank control is related to the number of reducing moieties present in the sample.

A standard curve was constructed using known amounts of glucose and the number of reducing terminals in the solution extrapolated from this standard curve.

4.2.12 Partial Nitrous Acid Degradation of Heparin

Low molecular weight heparin was obtained by partial nitrous acid degradation at pH 4. Nitrous acid was produced in situ by the addition of acid to sodium nitrite solution. The concentration of the nitrosting reagent was allowed to build

up for about 10 minutes before heparin was added. The following is a typical procedure used.

10 gm of purified heparin was dissolved in 75ml of 0.2 M sodium acetate buffer adjusted to pH 4. Nitrous acid was produced in situ by mixing 20 ml of a 4M sodium nitrite solution with 15 ml of the acetate buffer (pH 4). The nitrosating mixture was allowed to stirr at room temperature for exactly 10 minutes. The pH of the mixture was kept at pH 4 by intermittent addition of acetic acid. 75 ml of the heparin solution was added to the brown fuming nitrosating reagent mixture and stirred at room temperature for exactly another 10 minutes. The reaction was quenched by the addition of 7% ammonium sulphamate solution and the pH adjusted to 7 with saturated NaOH solution. Heparin was then precipitated out with excess cold ethanol. The precipitate was collected by centrifugation and redissolved in water.

The solution was then purified by passing the solution through the sephadex G-25 column twice to remove excess salt and small saccharide fragments if any. The sample was then freezed dried and analysed for free amines, reducing terminals and molecular weights according to the procedure outlined above.

4.2.13 Amination of Heparin

The C_1 carbons on reducing sugars can be made to react with primary amines to form a Schiff's base. The Schiff's base can be specifically reduced by cynoborohydride producing an imine. If the primary amine used in the above is a diamine the reducing terminal will be modified to carry a free amine group. This is the principle used to aminate the heparin molecule and the following is an example of the procedure used.

4 gm of heparin (whole or nitrous acid degraded) was dissolved in 15 ml of water. To this solution was added 15 ml of 20% hexadiamine solution. The mixture was adjusted to pH 10 or 7 and stirred for twenty minutes at room temperature. 2 gm of solid sodium cyanoborohydride was added to the mixture and stirred for 24 - 72 hours. At the end of the reaction the pH of the mixture was adjusted to 7 and filtered through a Schleicher and Schuell filter paper number 576. Sodium chloride was added to the filterate such that salt concentration in the filterate is about 1M. The filterate was then passed through sephadex G-25 column with 1M NaCl as the mobile phase to remove excess hexadiamine. The eluent was passed through the column a second time with pure water as the mobile phase to desalt the solution.

To remove traces of diamine the desalted heparin solution was passed through a column of cation exchanger resin (sodium form of Dowex 20). The sodium form of the

cation exchanger was obtained by passing excess NaCl (3M) through the column till the eluent is neutral.

4.2.14 High Voltage Paper Electrophoresis

The samples were spotted on a Whatman 3 paper (2ft by 4 ft) along a horizontal line in the middle of the paper that was prewet in 1% borate buffer (pH 9.0) and allowed to drip to remove excess water before sample application. The paper was slung over a rack at its middle and placed in the high voltage chamber containing borate buffer (pH 8). The electrophoresis was carried out at 1700volts for 30 - 45 minutes. The paper was then air dried before spraying it with 0.2% ninhydrin solution in ethanol and baking the paper in the oven for 15 minutes at 90^{o}C to develop the colour. In the presence of a primary amine the ninhyrin will give a purplish colour. Positively charged molecule will migrate in the opposite direction of negatively charged molecule away from the center of the paper where the samples were spotted.

4.2.15 Drying of the Hydrogel

PVA hydrogels was broken up into smaller pieces in a Warring blender. The gels was then solvent-exchanged with

increasing concentrations of acetone in water, viz. once each in 30%, 50%, 70% and 90%, and thrice in pure acetone (i.e. 100%). The time taken for each exchange is 24 hours. The gels dried in a vacuum oven at 60^{o}C for at least 72 hours. Homogenous fractions of the gel was obtained by passing the dried gels through a portable sieve shaker (Model RX24, C.E. Tyler) with a series of sieves of known mesh sizes. The various fraction was collected and kept in a dessicator in the dark till used. The fractions used in all this thesis was those between 45 -90 um in size when dry.

PEO hydrogel was ground manually with a razor blade and a mortar. It was then air dried in a vacuum oven at 50^{o}C for 72 hours or till its weight was constant. Then it was left to swell in dry THF for 4-7 days to remove traces of water in the gel. The THF was changed at least once a day during the swelling process.

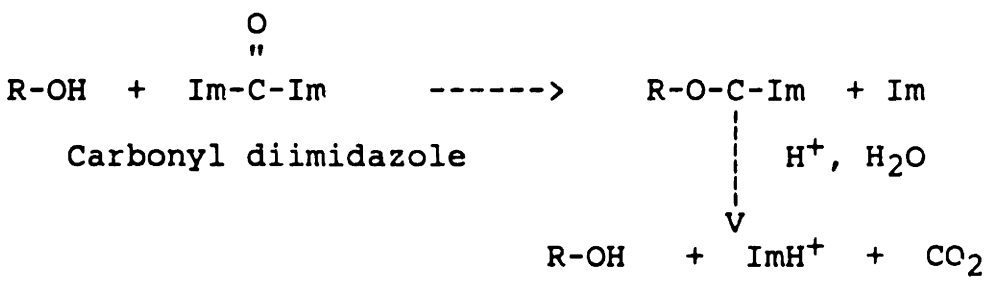
4.2.16 CDI Activation

A known weight of dry gel was allowed to swell in a dry dimethylformamide for 24 hours. A solution of carbonyldiimidazole in the same solvent was then added to the wet swollen gel and shaked gently for one hour. Excess unreacted CDI was washed off with more dry solvent. The activated gel was used immediately.

4.2.16.1 Determination of carbonyl imidazole on the gel

The CDI activated hydrogel was placed in water and the pH adjusted to pH 3 with HCl. The pH of the mixture was maintained at this level by adding HCl intermittently. The activated gels was stirred for 4-6 hours till hydrolysis of the activated group was complete. The mixture was then bubbled with nitrogen gas for 10 minutes to remove dissolved carbon dioxide. The imidazole released on hydrolysis was titrated with standard NaOH till the pH is greater than 9. Nitrogen gas was continuously bubbled into the mixture throughout the titration to minimise the effect of carbon dioxide on the pH of the mixture. The amount of imidazole released calculated from the milliequivalent of NaOH used to change the pH of the mixture from 4 to 9.

Standard solutions of imidazole was titrated with the standard NaOH. The moles of activated groups present based on the amount of imidazole released was obtained from this standard curve. The reactions involved are schematised in Figure 4-1). Appendix D shows an example of the titration curve of imidazole with NaOH.



$ImH^+ + OH^- \text{-------} > Im + H_2O \qquad pKa = 6.9$

Figure 4-1: Hydrolysis of activation group

4.2.16.2 Effect of CDI activation on the swelling of PVA

The activated gels were washed with pure water for about one hour with frequent changes of water. They were then allowed to swell in water for another 3 hours. The water was then filtered off with a vacuum and the weight of the wet gel obtained.

The gel was then dried in a vacuum oven at 60°C for 72 hours or till the weight reached a constant value. The volume fraction of the polymer was then calculated as outlined above.

The above was repeated with the CDI activated gels but with an additional treatment. The gels were deactivated by incubating it in 2% ethanolamine solution at pH 9 for 2 hours. Excess ethanolamine was washed off and the gels were then swelled in water.

Similarly, the volume fraction of PVA in the gels before activation, after activation and after deactivation in DMF was determined.

4.2.17 Activation by 2,2,2-Trifluoroethanesulphonyl Chloride

4.2.17.1 PVA activation by tresyl chloride

The dried PVA hydrogel was allowed to swell in dry DMF for 1 hour at 60°C and another 24 hour at room temperature. The solvent was then filtered off and fresh DMF added so that the gel slurry can be stirred by a magnetic stirrer. The amount of tresyl chloride added to the gel varied from 0.5mM to 1mM tresyl chloride per gm of dry PVA hydrogel. Pyridine acted as a base to neutralise the acid released on activation. The relative amount of pyridine to tresyl chloride used throughout the experiment was in the ratio of 2:1. Dry pyridine was added to the above slurry with stirring. Then tresyl chloride was added drop wise into the hydrogel slurry. Stirring was continued for ten minutes at room temperature all together. Unreacted tresyl chloride was removed by washing the gel with 100%DMF, 70%, 50%, 30% DMF/3mM HCL solution sequentially followed by 3mM HCl(200ml of each solution). The activated gel was used immediately or stored in 3mM HCl at 4°c for future use.

4.2.17.2 PEO activation by tresyl chloride

Activation was carried out in a 9:1 mixture of THF and DMF as the latter increased the solubility of tresyl chloride in THF. The activation was carried out at 4°C as the primary OH group of PEO is more reactive than that of PVA. 1.5mM of tresyl chloride was used per 0.5gm of dry PEO. The activation, washing procedure was similar to that for PVA except for solvents and temperature used.

4.2.18 Determination of Tresylates on the Hydrogel

The degree of activation was calculated from the flourine and sulphur content of the gel. The activated gel was washed extensively in 3mM HCl and freeze dried. The dried samples were then sent to Galbraith Laboratories (Tennessee) to be analysed for its sulphur and fluorine content.

4.2.19 Coupling of Heparin

Heparin was dissolved in 0.02M carbonate buffer at pH 10 to make a 5% solution. The activated gel suspension was washed with the carbonate buffer to remove the HCL present and the heparin solution added. The coupling mixture was allowed to shake at room temperature or 4°C for 24 hours. The excess heparin that was not bound was removed by repeated washing in 0.5M salt solution followed by water. Washing was stopped when the control (unactivated PVA) gel gave a

blue color with Azure A. The washed hydrogel was then allowed to sit in a small amount of pure water overnight. The water was then tested for the presence of heparin using the Azure A assay.

^{35}S-heparin was used to check that the above washing procedure was adequate. It was found that when the hydrogel was allowed to sit in the radioactive solution for 24 hours and then subjected to the same washing procedure as above, no radioactivity was found either in the wash or in the hydrogel indicating the washing steps above is adequate.

The washed gel was then deactivated by ethanolamine. 2% of ethanolamine at pH 10 was added to the washed gel and allowed to shake for 4 hours at room temperature. The gel was then washed and freeze dried.

4.2.20 Comparison between the reactivity of the hydroxyl and the amine group in the coupling

^{14}C-serine (specific activity 4,408,500 cpm/mM) and ^{14}C-glycerol (specific activity 3,671,840 cpm/mM) were bound to the hydrogel by the same procedure as for heparin. The gel was washed till no radioactivity was detected in the washed solution after the gel was allowed to sit in the same solution for 24 hours. The same procedure used to wash away excess heparin was used.

0.5 gm of the wet coupled gel was then counted in the liquid scintillation counter with the scintillation cocktail containing 20% water.

The stability of the bound ligand was determined by incubating the 1 gm of coupled hydrogel in each of the following at 37oC:

-0.02M phosphate buffer saline, pH 6.0

-0.02M phosphate buffer saline, pH 7.4

-0.02M carbonate buffer, pH 8.5

-citrated human platelet rich plasma containing 0.1% azide.

The gels and the above solutions were placed in 250ml polypropylene tubes and shaken gently on a rocking bed in a 37^{o}C environmental room. At fixed time intervals 0.4 ml of the slurry was removed and passed through a 0.45um disposable Millinex filter (Millipore Corp.). 0.2 ml of the filterate was pipetted and counted in the LSC in Aquasol scintillation liquid (New England Nuclear).

4.2.21 Stability of bound Heparin

^{35}S-heparin (1,161,290 cpm/mg) was coupled to the hydrogel via tresyl chloride as outlined above. The coupled gel was then incubated in citrated whole human plasma preserved with 0.1% sodium azide. The leaching of radioactivity into the plasma was assayed as above.

4.2.22 Heparin activity in solution

Assays of heparin anticoagulant activity were carried out by the 2 stage amidolytic method. Residual human thrombin was measured by its ability to hydrolyse the chromogenic substrate H-D-Phe-Pip-Arg-pNA (S2238). The rate of hydrolysis of the substrate by thrombin was monitored using the Lambda 3 UV/VIS spectrophotometer (Perkin Elmer) controlled by the IFL3 software from the PE 3600 data station at an O.D. of 405nm. The readings were taken every second and analysed with IFL3 software. The commercial heparin preparation from Diosynth B.V., Chicago, was used as standards by making appropriate dilutions and its activity taken to be its U.S.P. label value i.e. 158U/mg. A summary of the modified assay used is described below.

The water jacketed cell holder of the spectrophotometer was kept at 37°C by circulating water from the water bath (Precision Instruments) set at that temperature. A submersible magnetic stirrer was placed in the said water bath with a testtube rack attached to it. The contents of the reaction container was kept at 37°C at all times and stirred by placing a magnetic bar into each container and incubating it in the bath.

Phosphate buffer saline (0.025M phosphate, 0.125M NaCl, 0.01% azide pH 7.4) was used to make up all the stock

solutions. 0.02ml of heparin solution and 0.18ml of PBS was incubated in the water bath for 5 minutes. 0.3ml of thrombin solution (9U/ml) was added and incubated for 15 seconds. Then 0.5ml of antithrombin solution (27.5ug/ml) was added for 15 seconds followed by 0.1ml of polybrene (2mg/ml) for another 15 seconds. Then 0.2ml of S2238 (1.25mg/ml) was added and the contents of the reaction vessel immediately poured into a quartz cuvette preincubated at 37°C in the cell chamber of the spectrophotometer. The change of absorbance with time at 405nm was followed for 60 seconds using the data station and the IFL3 software. The rate of hydrolysis of the substrate as seen by the rate of change of absorbance at 405nm was calculated using the Kinetic program of IFL3. A typical reading of the output is given in Appendix E. All assays were done in triplicates. The activity of the unknown heparin was calculated from the standard curve using the commercial heparin.

4.2.23 Activity of Bound Heparin

The above method for determining heparin activity in solution was modified to assay for the activity of the bound heparin. The standard heparin activity curve was constructed using heparin solution of known activity with pure PVA hydrogel in it. 3mg of dry PVA hydrogel was added to each tube and 0.2ml of PBS added and the tube tightly sealed. The

gel was allowed to swell in the PBS for 48 hours before the assay.

The heparin solution was added to the PVA just before the assay to minimise the amount of heparin penetrating into the gel matrix. The hydrogel was filtered from the enzymatic mixture after the addition of polybrene solution using an Econo-column (Biorad) fitted with a glass fitted filter at the lower end before the addition of the substrate. Filteration was accelerated by using air pressure so that filtration takes less than 5 seconds. An example of the procedure used is as follows:

3 mg of Hep-PVA (PVA for the standards and blank) was allowed to swell in 0.4ml PBS (0.2ml for the standards) for 24 hours. They were then incubated at 37^{o}C for 5 minutes with constant stirring with the magnetic stirrer. For the standard curve 0.2 ml of the standard heparin solutions were added to the standards just before the assay. 0.3 ml of thrombin was added for 15 seconds followed by 0.5 ml of antithrombin for 15 seconds. Then 0.2ml of polybrene was added. After exactly 15 seconds the reactants was immediately filtered through the econo columns under slight air pressure. 0.8ml of the filterate was removed and added to another clean tube, 0.2 ml of S2238 added and the reactants transferred to the quartz cuvette and the rate of change of O.D. at 405nm taken with the IFL3 software on the Perkin Elmer Data Station for 60 seconds. The tubes and columns

6 RESULTS AND DISCUSSION

6.1 Crosslinking of PVA in solution

6.1.2 Molecular weight of PVA

The molecular weight of the PVA sample used was analysed from the universal calibration curve constructed from PEO narrow molecular weights. Of the molecular standards commercially available PEO resembles PVA most closely as they are both soluble in water and essentially linear in structure. Figure 6-1 shows the universal calibration curve constructed from the the PEO standards and the different molecular weights of PVA are:

Number average molecular weight - 43,000

Weight average molecular weight - 110,000

Viscosity average molecular wt. - 105,000

$$\frac{M_w}{M_n} = \frac{110,000}{43,000} = 2.56$$

The concentration of the PVA solution used in the analysis was 0.1% to limit self association that is commonly seen in higher concentration of PVA solution. The viscosity average molecular weight corresponds very closely with that supplied by the manufacturer (Polysciences PA) which was 108,000. Therefore it was assumed that the number and weight

average molecular weight obtained by using PEO standards is the correct value.

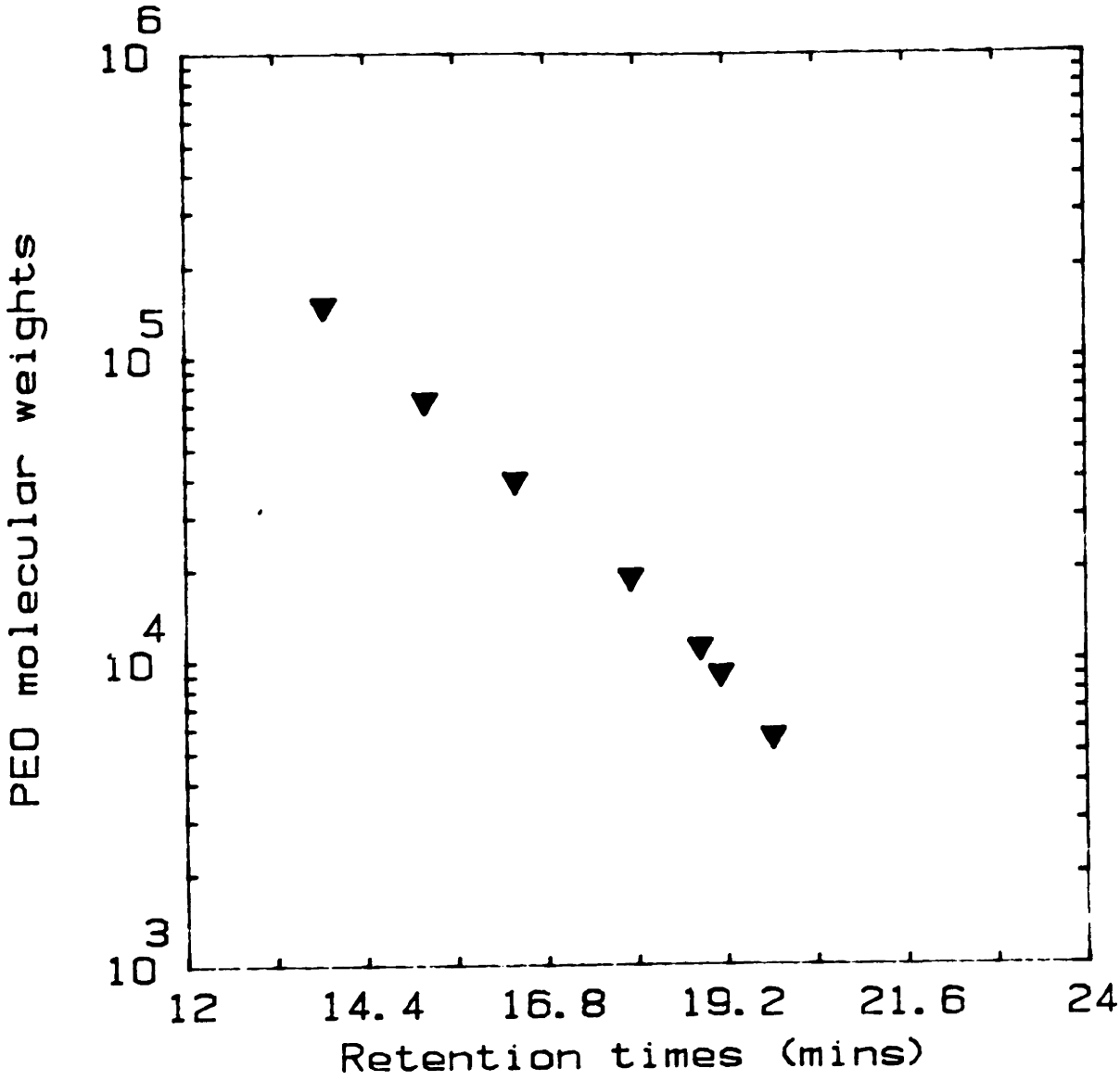


Figure 4-1 : Universal calibration curve for SEC. The PEO standards were run in water at 30°C on Toyosoda columns TSK G5000PW, G4000PW,AND G3000PW

The SEC result showed that the PVA sample is polydispersed with a most probable molecular weight distribution.

6.1.2 PVA Hydrogels

The hydrogels produced by saturating the PVA solutions with argon prior to irradiation were transparent and practically free from air bubbles without going through the freeze-thaw process of Peppas(1973). Saturation with argon and deaerating the solution removed most of the hydrogen, nitrogen and carbon dioxide besides oxygen from the solution. Argon is only very slightly soluble in water. On irradiation, the temperature of the solution/gel could increase from the room temperature of about 20°C to about 40°C. The atmosphoric gases, being less soluble at higher temperature were released and contributed to the formation of gas bubbles found in Bray's hydrogel (1972).

Figure 6-2 shows the effect of radiation dose on the average molecular weight between junctions (M_c) of PVA hydrogel made from a 5% and a 10% solution. For both cases, M_c initially decrease sharply with dosage and then levels off at higher doses. The same data was replotted as $1/M_c$ versus dose as in figure 6-3. M_c is inversely proportional to dosage although the relationship is dependent on the initial concentration of the solution. The rate of decrease in M_c is higher at the higher PVA concentration. At higher polymer concentration, the concentration of macroradical at

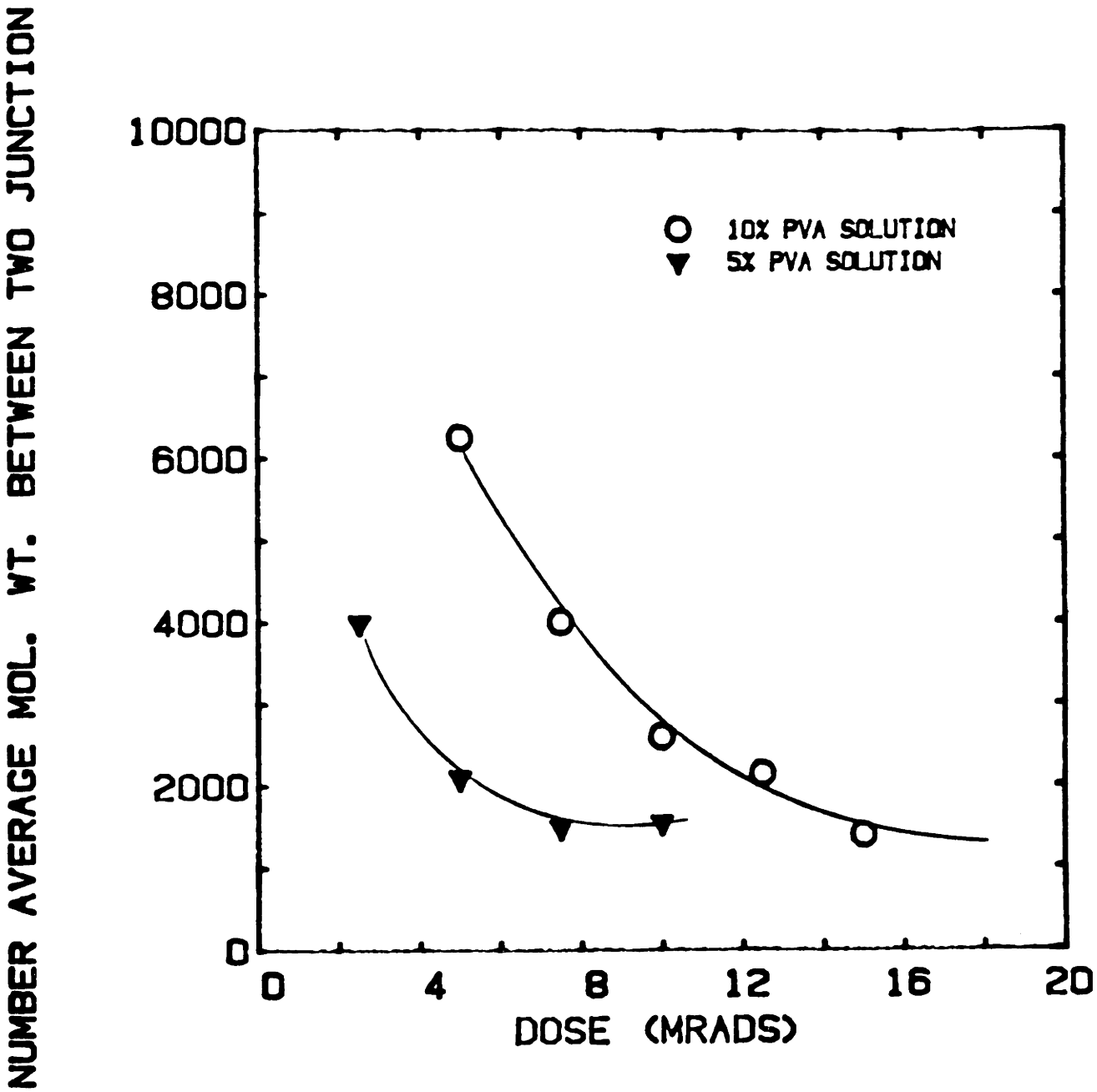


Figure 6-2: The interjunction number average molecular weight as a function of dose.

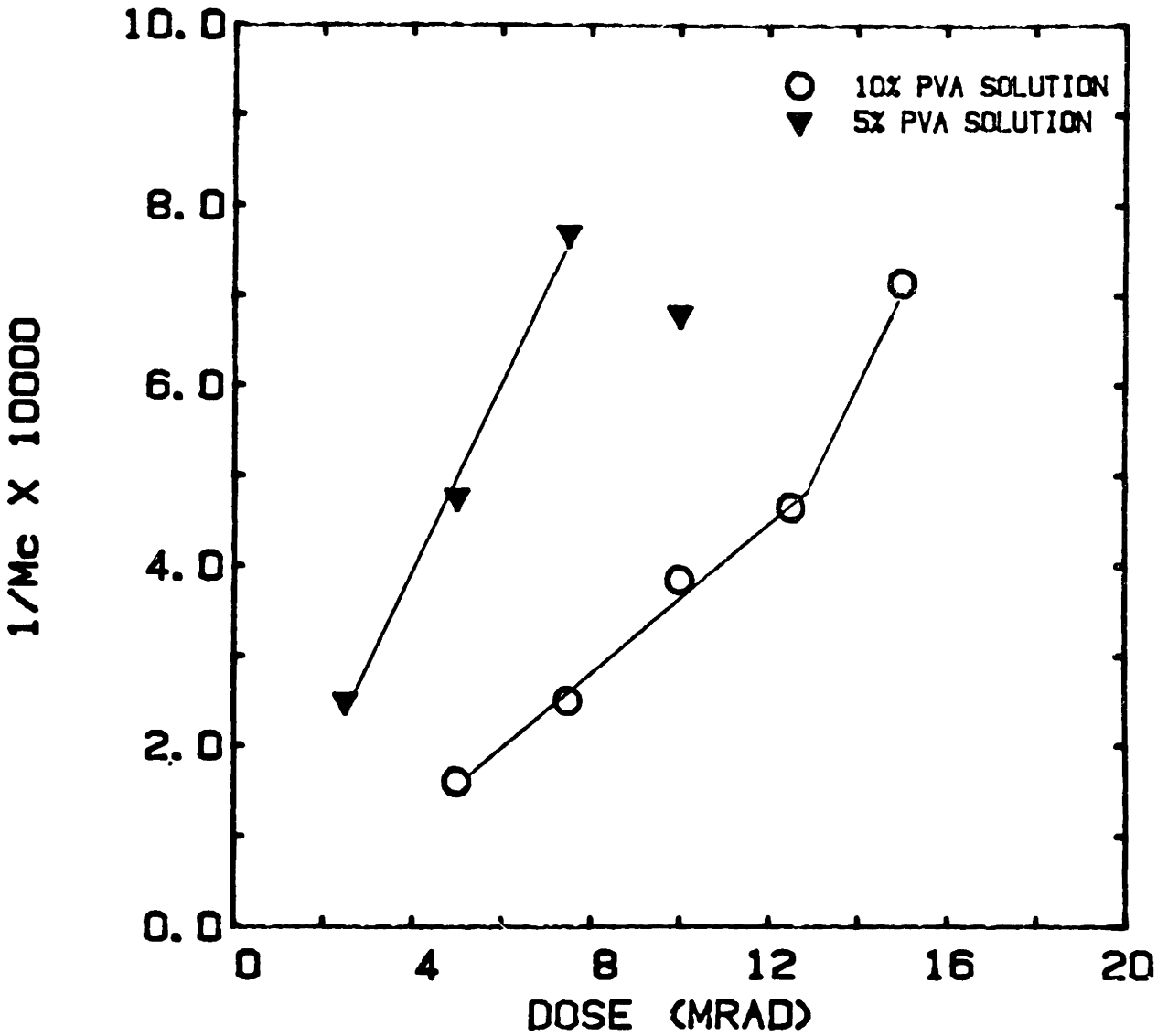


Figure 6-3: $1/M_c$ versus dose

any one time is higher thereby increasing the efficiency of the crosslinking process. This effect is enhanced in PVA solutions as the polymer has a high tendency to associate together by hydrogen bonding and adopting some form of preferred conformation (Sakurada and Ikada, 1961).

6.1.3 Penetration of Globular Protein into the Gel

The effectiveness of the various gel to exclude proteins is listed in table 6-1. Gels made from the 5% solutions would allow proteins as large as or smaller than BSA to penetrate into its matrix even with a M_c of 1500. On the other hand the hydrogels made from the 10% solution with a M_c of about 2000 could exclude even chymotrypsinogen whose molecular weight is 23,000. This same behavior were reported by Bray(1972) and Bauer(1972) but the authors did not offer a satisfactory explanation. It appears that the 'molecular pore size' of the hydrogel is not a function of M_c value alone but also depends on other parameters as elaborated below.

For the sake of clarity two representative gel made from each concentration will be used to illustrate the cause of the anamolous behavior. The chains in the gel are in constant fluctuating motion all the time. Hence it would not be a real barrier to the penetration of the protein into the

Table 6-1 : Penetration of Proteins into PVA hydrogels

PVA %	Dose (Mrad)	M_c	BSA	Ovalb	Chymotryp.	lysozyme
5	2.5	4000	y	y	y	y
	5.0	2100	y	y	y	y
	7.5	1300	y	y	y	y
	10	1400	y	y	y	y
10	5	6250	y	y	y	y
	7.5	4000	n	n	y	y
	10	2600	n	n	n	y
	12.5	2150	n	n	n	y
	15	1400	n	n	n	y

gel. The motion of the junction points on the other hand is much more restricted and can be considered to be more or

less fixed in space. The junction points then act as a mesh to the challenging protein. The size of the mesh is determined by the nearest neighbour distance between junctions (d_s) and not the topological distance ($\langle r^2 \rangle^{1/2}$) as calculated from the M_c value. The calculated value of the two distances are given in table 6-2 for the two gels the M_c of both being approximately the same.

For both the gel the value of d_s is large enough to allow BSA (R_g = 36 ^oA) through yet only the 10% gel will exclude BSA and even chymotrypsinogen (R_g = 22^oA). For a totally homogenous gel like PEO the pore size will probably depend almost entirely on the value of d_s. Concentrated solution of PVA however have been observed to exhibit supermolecular order (Klenin et al.,1966) at temperatures below 125^{o}C. These supermolecular structures are small aggregates of PVA chain segment held together by intermolecular hydrogen bonding forming some kind of paracrystalline structures. The aggregates are very small and therefore they do not scatter light and hence the solution or gels where they are present will appear to be transparent. The size of these supermolecular structures could be increased by freezing the solution. The larger structures are now large enough to scatter light and the solution are no longer transparent. This phenomena was documented and described in Peppas' thesis (MIT 1973).

Table 6-2 : Average distances between junctions

	5% PVA	10%
Dose (Mrad)	5	12.5
M_c	2100	2150
Equil. vol fraction(V_{2s})	0.067	0.110
Ave. Spatial dist.; d (^oA)	43.4	36.8
Ave. Topological dist.; $\langle r^2 \rangle^{1/2}$ (^oA)	87	74

Note: Characteristic ratio used for calculating the topological distance was 8.5 (Wolfe and Suter, 1984)

In hydrogels, the same supermolecular structures could be formed under suitable conditions. Due to the shorter average distance between junction in PVA hydrogel's chain length and the more restrictive mobility of the chain in the gel, a higher concentration of chain segments will be required before these structures will be present in significant quantities. It is concievable that the supermolecular structures were present extensively in the 10% gel but may not be found in the 5% gel or if present were found only in small amounts.

These paracrystalline supermolecular structures then can act as extra 'junctions' in the gel matrix making the gel-mesh tighter than that calculated from the junction density in the 10% gels accounting for the ability of these gels to exclude much smaller proteins than would otherwise be possible.

For the purpose of this thesis, the gel matrix should be able to exclude both antithrombin (M.W. = 58,000) and thrombin (M.W. = 38,000). The hydrogel that will exclude chymotrypsinogen was considered tight enough for this purpose it being smaller than either of the proteins.

6.1.4 Heparin Penetration into the hydrogel

Figure 6-4 shows the change with time in the heparin concentration of the heparin solution into which a known

volume of hydrogel was placed. The concentration of the heparin solution remains unchanged even after 6 days of continuous scaking. This implies that the heparin molecule was not able to penetrate into the matrix and therefore any bound heparin found after the coupling was restricted to the surface of the hydrogel. This is not surprising as the hydrodynamic size of heparin as measured by size exclusion chromatography was found to be between that of fibrinogen (420X50 A) and bovine serum albumin (36 A). Similar results was obtained when the gel was challenged with nitrous acid degraded gel. The very small molecular weight fraction of the degraded heparin may have penetrated into the matrix but could not be detected experimentally.

6.2 Characterisation of Commercial Heparin

Table 6-3 list all the type of heparin purchased and prepared for this study. All the heparin were from Diosynth, Chicago and the modification were made using the purified commercial heparin.

6.2.1 Commercial Heparin (PH and CPH)

Size exclusion chromatography of the heparin samples using solutions of various ionic strength (pure water, 0.05M, 0.1M, 0.2M NaCl) showed that in all cases there was only a single peak and that the species present were very sensitive to the ionic strength of the solvent as a result of the high charge density of

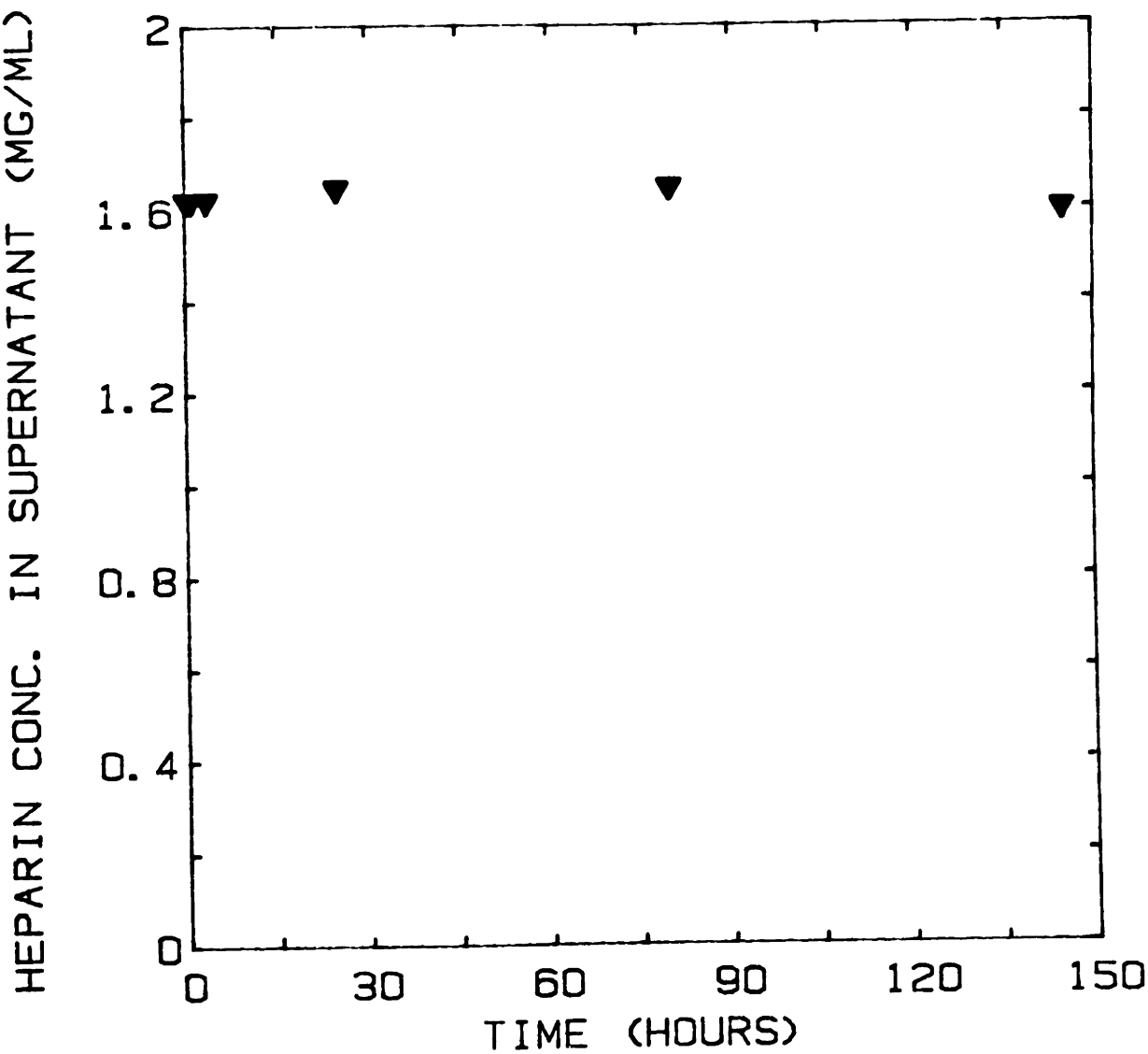


Figure 6-4: Concentration of Heparin in the supernatant as a function of time. Volume of heparin solution is 1.5ml while the total volume of moisture in the PVA hydrogel was 1.25ml in each vial

the molecules. Since there are no other natural polysaccharide with such a high charge density the above sample was assumed to contain only heparin. Results from the amino acid analysis showed negligible amounts of protein in the sample .

Table 6-3 : Types of Heparin Preparations

CODE NAME	SOURCE/PREPARATIONS
PH	Commercially purified heparin
CPH	Crude heparin purified in our lab by repeated ethanol precipitation and filteration
PH7A	PH heparin modified by attaching hexadiamine to its reducing termini at pH 7. Reaction was carried out for 24h.
PH7B	As in PH7A but reaction allowed to continue for 72 h.
PH10	PH heparin modified by attaching hexadiamine to reducing terminal at pH 10. Reaction time was 24h.
MHNO	PH heparin that has been degraded by nitrous acid at pH 4
MHNH	MHNO that has been further modified by attaching hexadiamine at pH 10

The number and weight average molecular weight of both PH and CPH were similar and were 6300 and 7000 respectively as determined by size exclusion chromatography. It is to be noted here that the molecular weight is relative to the standards used. These standards were prepared by partial nitrous acid degradation and purified by column chromatography. The standards are rather small and covered only a small range of molecular weight. Therefore the molecular weights of the heparin quoted here and elsewhere in this study are to be used only as a gauge to scale them relative to each other. The molecular weight quoted by the manufacturer using dextran standards for pure heparin was 20000 compared to 7000 obtained using the Choay standards.

Their amino acid composition is given in table 6-4. The amino acid content of both the preparation were quite similar. The amino acid content that are of interest to this study is the serine and glycine content. Serine being found at some of the reducing terminal of the chain and glycine will give us an indication of how much of the backbone protein remained intact after the purification process.

The amount of serine in PH and CPH were 22 umole/gm and 25.7 umole/gm respectively. For a M_n of 6300, 14% of PH chains and 16% of CPH chains has a serine residue which reside at the reducing end (Rosenberg, 1982). This assumes that there is only one serine residue at each terminal. This

Table 6-4 : Amino Acid content of Heparin

Amino acid(um/gm)	Pure Heparin	Crude Heparin
SCM	0.59	1.9
Aspartate	-	2.2
Threonine	-	1.98
Serine	22.22	25.68
Glutamate	0.38	2.38
Proline	0.79	2.16
Glycine	1.34	6.38
Alanine	-	1.56
Valine	-	0.61
Histidine	2.93	2.3
Lysine	0.93	2.23

assumption is probably correct for PH but not for the CPH as the latter has not undergone as extensive purification as

the former. The extraction and purification of heparins involves extensive protease digestion. The digestion chops up the backbone to small peptides or single amino acids and the purification process removes these amino acids and oligosaccharide. The low amounts of glycine found in PH further supports this hypothesis as the proteoglycan backbone is believed to consist of large segments of alternating sequence of glycine and serine with the polysaccharide radiating from the serine residue. Presence of large fragments of the protein backbone will be reflected by the presence of larger amounts of glycine. The glycine content of CPH is about 5 times that of PH although this is not reflected in a corresponding increase in the serine content which implies that the glycine residues were adsorbed to the polysaccharide chain and the purification process used was inadequate. Therefore all subsequent studies with heparin was carried out with the commercially purified heparin.

The number of reducing ends are given in table 6-5. In theory the number of reducing ends together with the serine content of one gram of heparin should be equal to the total number of chains in one gram of heparin. Based on this assumption the number average molecular weight of PH and CPH will be 20,120 and 18,620 respectively which is much higher than the 6,300 obtained from the SEC measurements. This is partly due to the modification the reducing terminal have

undergone in the purification and extraction process where oxidation is commonly used or it may be because the heparin standards used was less than adequate. Unfortunately heparin standards other than the ones mentioned above were not available and using other polymers or polysaccharides as standards is not possible to the unique property of heparin carrying a very high charge density.

The unsubstituted glucoseamine content of PH and CPH obtained by subtracting the serine content from the total primary content are 20.7umole/gm and 31.4umole/gm respectively. If we assume that each chain contains only one unsubstituted glucoseamine, then approximately 13 percent of the PH chains will contain an unsubstituted glucoseamine. The distribution of this glucoseamine amoung the molecules is probably random. Some molecules may contain both the terminal serine and one or more unsubstituted glucoseamine(s) while others may contain either one of them or none altogether. This has large implication in selecting the chemistry to bind the heparin to a substrate. The carboxylic acid content of heparin is 2 orders of magnitude higher (Table 6-5) then the amine content. Binding of heparin via this group will result in the molecule being attached to the substrate along its backbone and at more than one point. The low primary amine content on the other hand is an advantage in that the chain is more likely to be bound at one position and hence decreasing the probability of deactivating the heparin

Table 6-5 : Analysis of Diosynth Heparin

	Pure Heparin	Crude Heparin
M_w	7000	7100
M_n	6300	6500
COOH (mM/gm)	1.26	1.40
SO_3H (mM/gm)	3.06	3.28
$COOH/SO_3H$	0.412	0.426
Total 1^o amine (uM/gm)[a]	56.7	71.7
Serine (uM/gm)	22.2	25.7
Unsubt. $gluNH_2$ (uM/gm)[b]	34.5	46.0[c]
Red. group (uM/gm)	27.5	28.0

a - measured by fluorescamine and glucoseamine as standard
b - assume that contribution to the amine content is by serine and unsubstituted glucoseamine along the backbone only
c - rather high value is due to presence of amino acid residue other than serine (see amino acid content in table 6-4

molecule although the yield of bound heparin is proportionately lower when the amine functional group was used.

6.3 Chemistry of Coupling Reaction

Activation of the PVA with tresyl chloride did not result in a significant change in the swelling ratio of the activated PVA hydrogel even at the highest activation used in this study. This is in contrast to the activation by carbonyl diimidazole (CDI) whereby the swelling ratio decreases continously as the degree of activation increases (Appendix D). Therefore it was chosen over CDI

6.3.1 Coupling of Serine and Glycerol to PVA

The reactivity of the amino and hydroxyl groups towards tresylated PVA were compared by using serine and glycerol to represent the two groups respectively. The coupling yield for serine was 71.3% while it was only 21.3% for glycerol (table 6-6) i.e. the amino group is about 3.5 times as reactive as the primary hydroxyls.

Glycerol was able to react with the tresylate because of the presence of two primary hydroxyls in the molecule; it being more reactive than the secondary hydroxyl. In glycerol, the attacking species is the alkoxyl ion (RO^-) formed by the dissociation of H^+ at high pH. The alkoxy group is however weaker acid than water: the relative acidities of the various group being:

H_2O > 1° alcohol > 2° alcohol > 3° alcohol

(Morrison and Boyd, 1969).

It follows from the above argument that the reactivity of the hydroxyls towards tresylate will follow the same hierachy as their acidities. This is born out by the low yield obtained - most of the tresylate being hydrolysed by water instead. The secondary hydroxyls of heparin is expected to have an even lower yield than glycerol.

The amino group of serine (pKa = 9.2) on the other hand was almost totally in the unprotonated form at the pH of coupling. This unprotonated form is the nucleophilic species that reacts with the tresylate. This gave a yield of 71%.

Heparin is therefore expected to be bound to the activated gel primarily if not solely through its primary amines.

Table 6-6 : Coupling of ^{14}C-Serine and ^{14}C-Glycerol to PVA

	Serine	Glycerol
uM treslate/gm PVA	122	122
uM bound/gm PVA	87	26
% yield	71.3	21.3

6.3.2 Stability of the C-N bond

The displacement of the tresylate by the amino function of the serine molecule produces a C-N bond(R_1-CH_2-NH-R_2). This bond was found to be resistant towards hydrolysis between the pH of 6 to 8.5 which encompasses the physiological pH range of all biological system in which the hydrogel will be subjected to (figure 6-5). The amount of serine leached into the supernatant even after 70 days of incubation was negligible as there was no increase of counts from day 1 to day 70 of incubation.

When incubated in platelet rich plasma (PRP), the amounts of count in the plasma increases slightly and the plateau off at about 7 % (figure 6-6). The initial increase in counts found was due to the adsorption of small amounts of serine that could not be washed off but was displaced by the plasma protein. The presence of enzymes that can hydrolyse the C-N bond would result in a more rapid rise in counts in the PRP.

The stability of the bond between the substrate and the amine containing ligand will allow the heparinised material to retain its biocompatibility indefinitely if the heparin is also stable under these conditions and remained active when bound.

6.4 Coupling of Pure Heparin to PVA

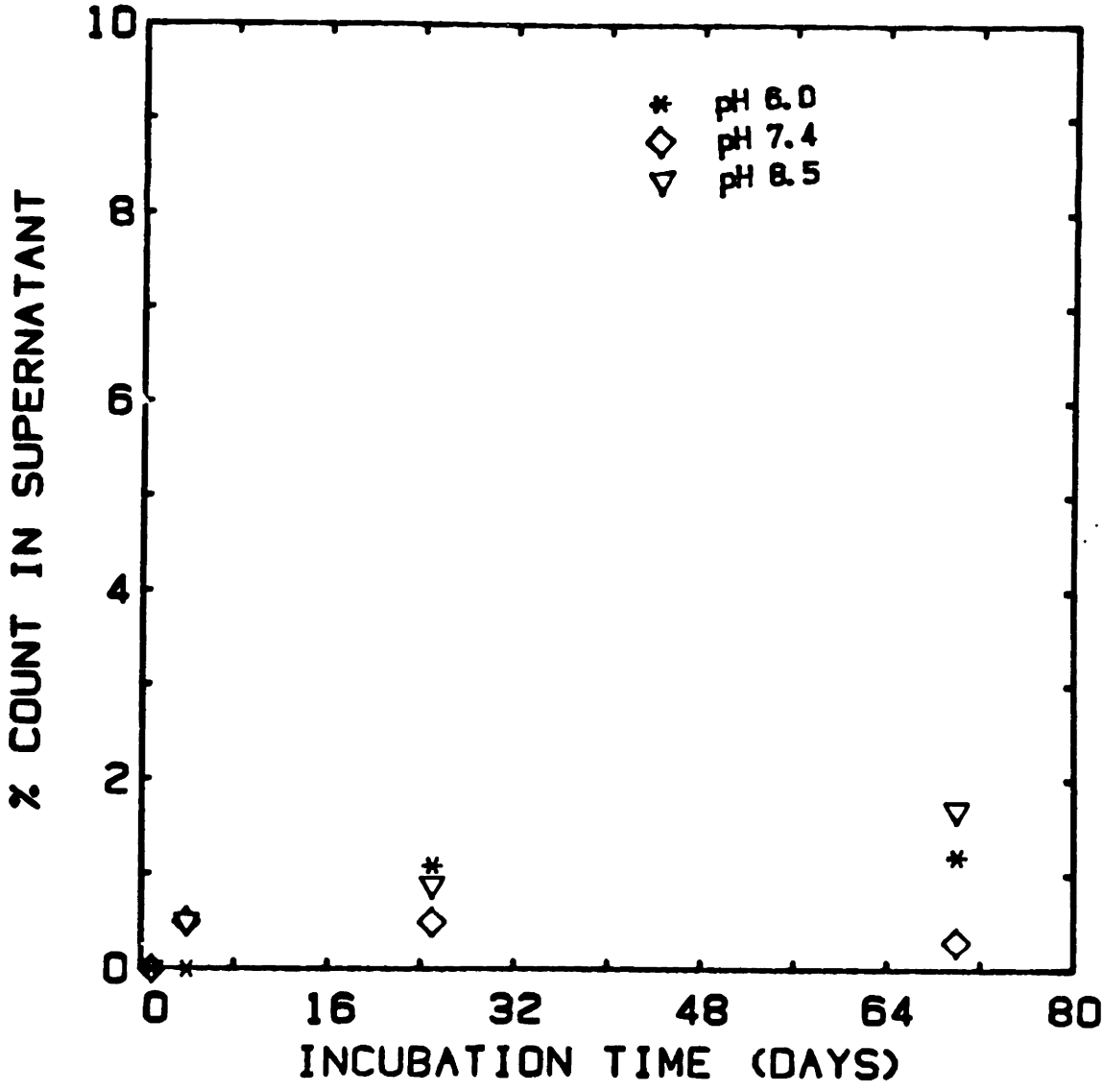


Figure 6-5 : Stability of Bound ^{14}C-serine as a function of pH at 37 ^{o}C

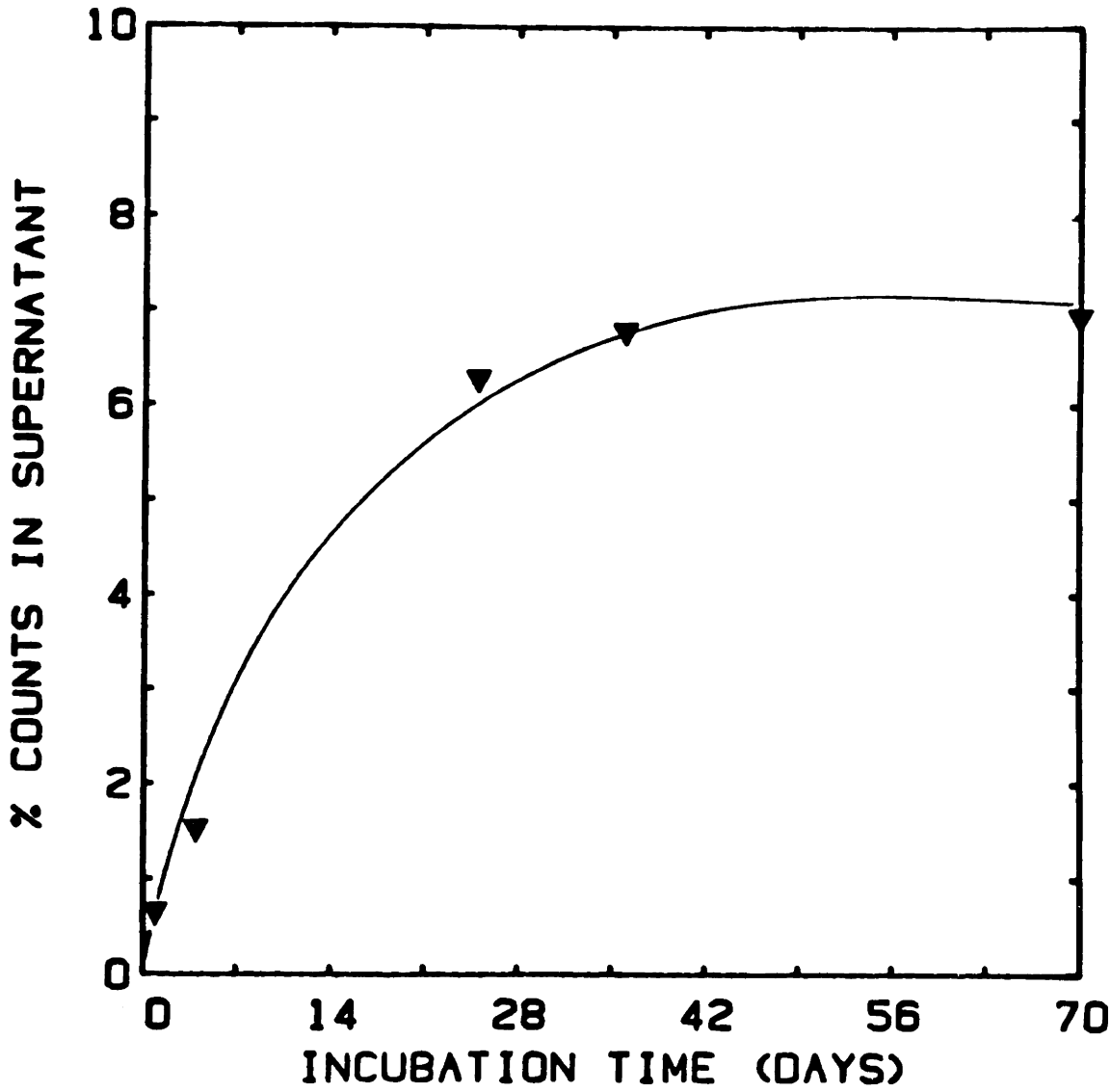


Figure 6-6 : Leaching of Bound ^{14}C-serine into platelet rich plasma at $37^{o}C$

Figure 6-7 shows that the amount of activated group on the PVA can be easily controlled by changing the ratio of tresyl chloride to PVA used. Unless otherwise specified all the PVA gels in the subsequent study has about 56uM of treslylate per gm of dry PVA.

6.4.1 Stability of Bound Heparin in Plasma

The bond formed between the heparin and the PVA hydrogel is stable in plasma as demonstrated above. However, heparin can be degraded along its backbone chain by glucosidases that may be present and hence losing its activity with time. Heparinase-like activity have been reported (Oldberg et al.,1980; Oosta et al., 1982) to be found in human platelets although no heparinase activity has been reported to be found in plasma itself. The heparitinase activity is believed to be due to the presence of endoglycosidases present within the platelet lysosomes. The role of these enzymes in the hemostasis of blood is unknown.

Figure 6-8 shows the rate at which ^{35}S-heparin were leached into the PRP. There was no significant increase in counts for the first five days of incubation at 37^{o}C. This confirms the absence of heparinase activity in normal blood plasma. The counts suddenly began to increase from the sixth day to the ninethenth day and then levelled off. Altogether

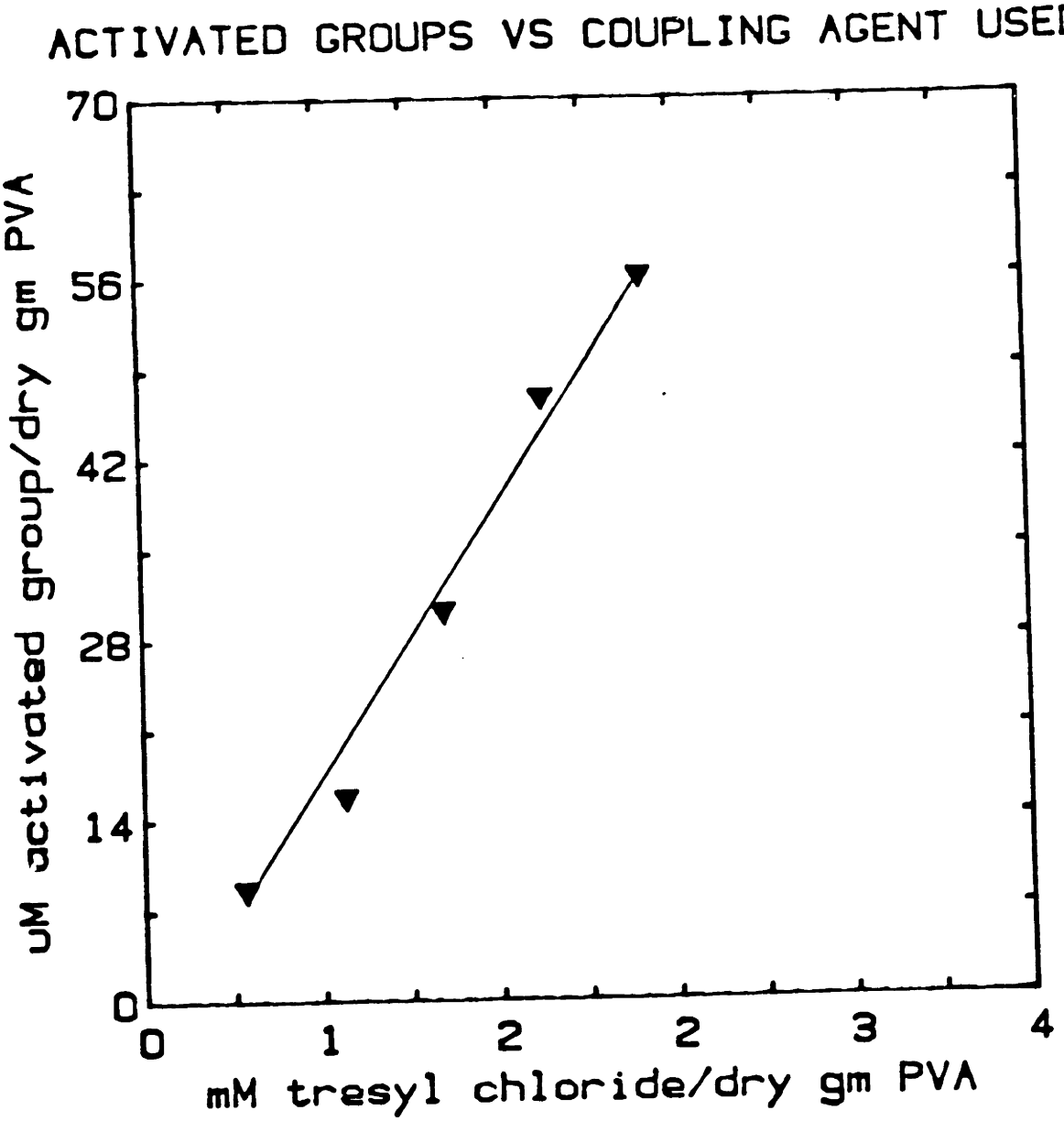


Figure 6-7 : Degree of activation as a function of coupling reagent added in DMF at 22°C

only about 14% of the bound heparin leached out into the supernatant.

Leaching of heparin into the plasma after the fifth day of incubation is attributed to microbial contamination -

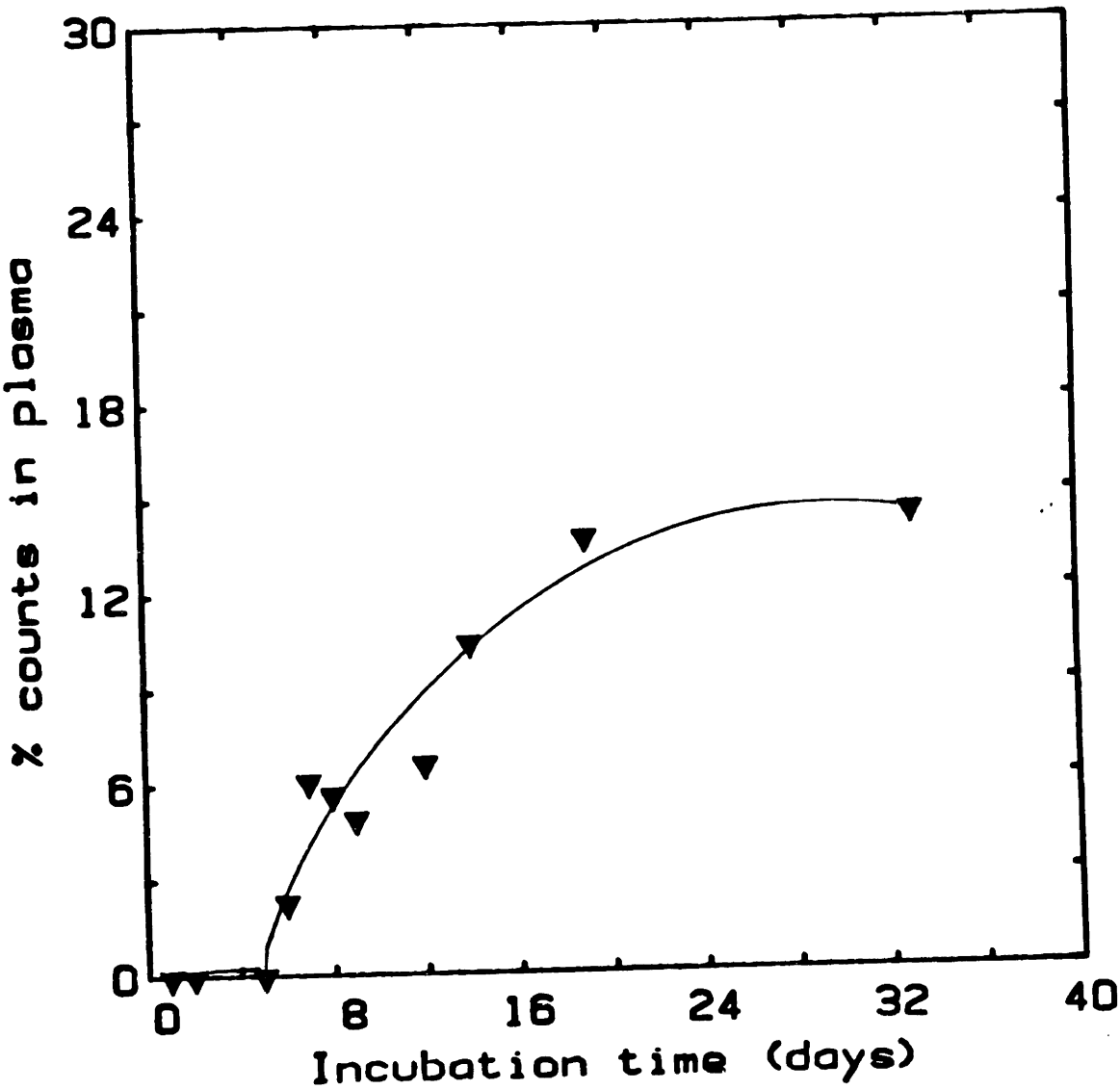


Figure 6-8 : Stability of bound ^{35}S-Heparin in Platelet Rich plasma at 37°C

enzymes especially exo- and endo-glucocidases produced by these microbes could degrade the bound heparin and release it into the plasma; the plasma being a very rich source of nutrients for microbial growth. Although precaution against contamination was taken by adding azide and using sterile pipette tips, the frequent sampling required could introduce some microoganism.

The plateauing of the count after 20 days of incubation could be accounted for by the fact that the configuration of the immobilised heparin may not be in the form where the heparitinase can attack it. Steric hindrances also will decrease the activity of the enzyme towards the bound heparin. In the actual application of the biomaterial the scenario presented above is not likely to be found and hence bound heparin will be stable in invivo systems since heparinase activity is absent in fresh plasma.

6.4.2 Effect of temperature on the Rate of Coupling of Heparin

The rate of binding of heparin to tresylated PVA was studied at 22°C and 4°C at pH 10. The amount of heparin bound was calculated from the sulphur content of the gel and the results are shown in figure 6-9 and 6-10.

At 4°C, the coupling reaction was completed in about 8 hours while coupling at 22°C required about 12 hours for the limiting extent of coupling to be reached. Further exposure

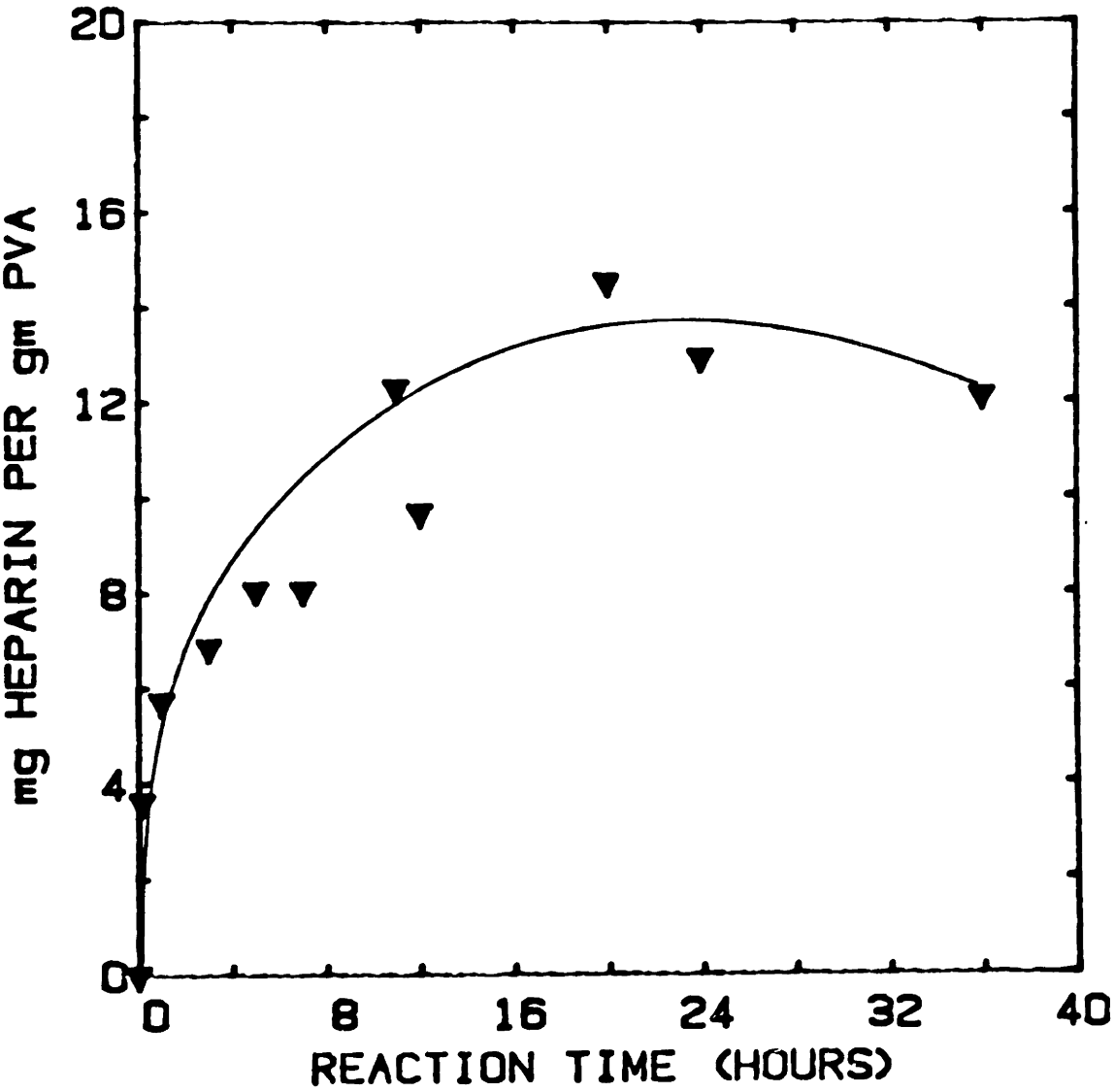


Figure 6-9 : Rate of coupling of Heparin to PVA at 22°C and pH 10. The PVA has about 50μM of tresylate per dry gramme of PVA

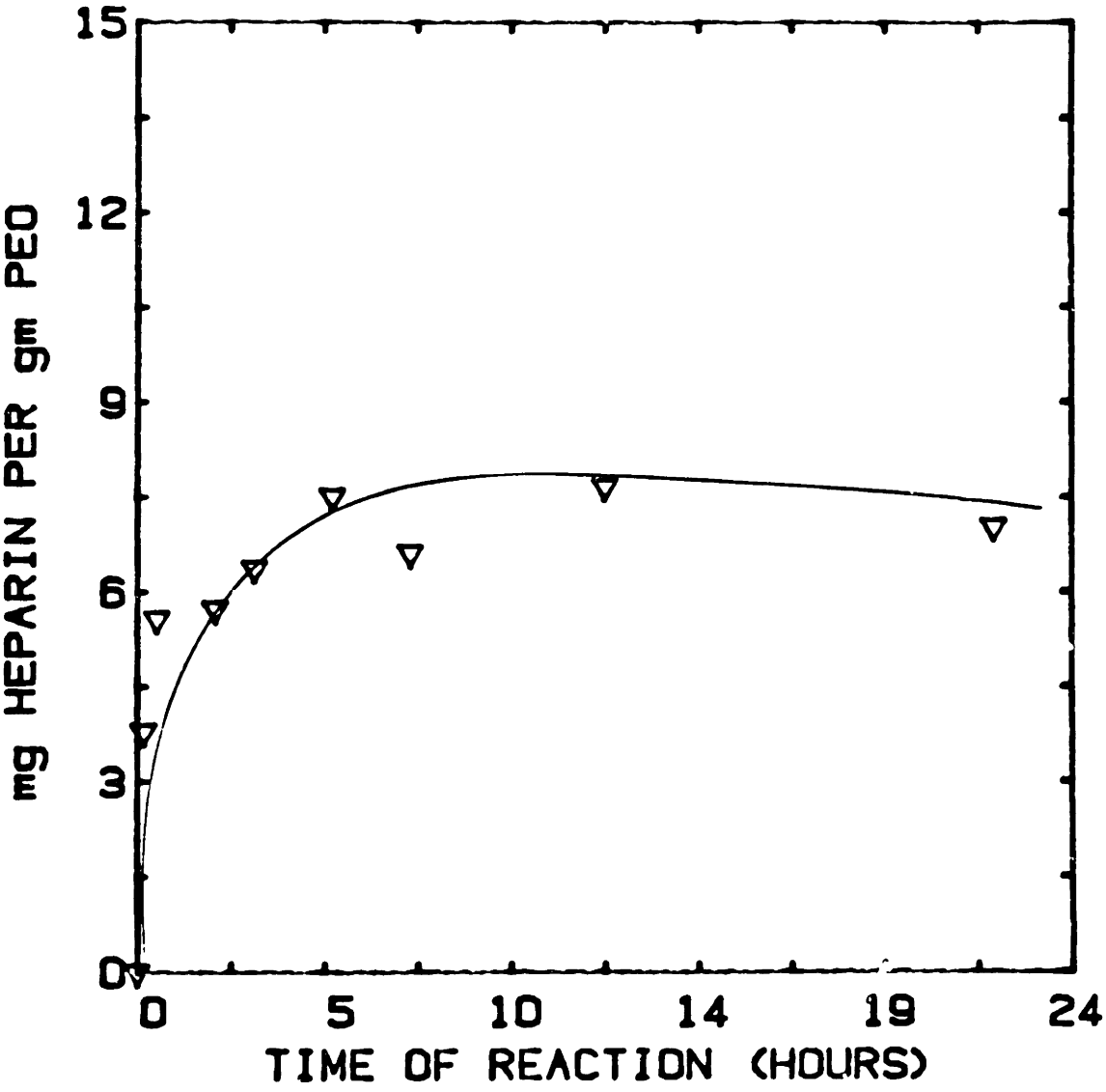


Figure 6-10 : Coupling of Heparin to PVA at 4°C and pH 10
There are about 50μM of tresylate per dry gm of PVA

to the heparin solution did not result in an increase in the binding of heparin to the polymer surface. The reaction however was allowed to proceed for more than 24 hours in both cases to complete the hydrolysis of unreacted tresylate if any.

The results show that the final amount of heparin bound is higher if coupling was carried out at a higher temperature (10mg at 22°C versus 3.5mg at 4°C). This can be explained by the fact that two types of reactions were occurring when the tresylated PVA was placed in the coupling media. One is the main reaction of the tresyl ester with the amine group of the heparin and the other the side reaction of the hydrolysis of the ester by water.

The coupling reaction may be more temperature sensitive than the hydrolysis reaction under the pH condition used. Reaction of the PVA hydroxyls with tresyl chloride produces only secondary tresylates. These tresylates unlike those derived from primary alcohols couples with difficulty (Scouten and van Der Tweel,1984). In other words its activation energy is relatively high. The hydrolysis of the tresylate under alkaline reaction on the other hand may be less temperature sensitive. The Arrhenius equation:

$$k = A \exp(-E_a/RT)$$

where

k = rate constant

A = constant

E_a= activation energy

R = universal gas constant

T = temperature in kelvin

shows that the rate constant of a reaction is more sensitive to temperature if its activation energy is higher. Therefore raising the reaction temperature from 4°C to 22°C will increase the rate constant of the coupling reaction more than the hydrolysis reaction giving a higher yield.

If one assumes that one molecule of heparin is attached to only one hydroxyl group of the PVA, then only about 0.007% of the available hydroxyls of PVA carries a heparin molecule (based on the yield at 22°C and M_n of 6300 for heparin). From this result and that from the studies on the penetration of heparin into the gel matrix it was concluded that the hydroxyls involved are mainly situated at the surface of the hydrogel.

Table 6-7 and 6-8 show the variation of heparin activity and specific activity with time of reaction. At 22°C the total activity found in the gel initially increase as the amount of heparin bound increased. After 7 hours further increase in heparin bound did not result in an increase in activity. The lost of activity with time is reflected more clearly in the specific activity of the bound heparin.

Table 6-7 : Rate of Heparin bound to PVA at 22°C and pH 10

Coupling time (hours)	Heparin (mg/gmPVA)	Tot. Act. (U/gmPVA)	Spec.Act (U/mg Hep)
1	2.096	5.133	2.45
3	4.43	9.46	2.136
5	4.43	8.31	1.876
7	6.05	9.81	1.62
12	9.68	8.54	0.88
24	9.3	8.3	0.89

Table 6-8 : Rate of Heparin bound to PVA at 4oC and pH 10

Coupling time (hours)	Heparin (mg/gmPVA)	Tot. Act. (U/gmPVA)	Spec. Act. (U/mgHep)
0.5	1.78	2.18	1.23
2	1.94	2.13	1.17
3	2.59	2.94	1.13
5	3.72	3.58	1.08
7	2.83	3.33	1.10
12	3.88	3.58	0.92
24	2.99	3.09	1.03

The lost in activity with time of coupling is probably due to the denaturation of the of some of the active heparin that was already bound. Figure 6-11 is a schematic diagram of how the pure heparin was probably bound to the PVA under the conditions used here. It is postulated that the activity of the bound heparin is mainly contributed by the molecules bound only at its serine terminal end. The molecules bound at only one point via the glucoseamine may or may not retain some of its original activity while those bound at multiple points is not expected to retain any of its antithrombotic activity. As the coupling time was allowed to increase the probability of a heparin molecule being able to react with two neighbouring tresylate increases causing a drop in specific activity of the bound heparin.

The activity of heparin bound at 4°C on the other hand was not affected as much when coupling was allowed to continue for 24 hours. The total activity continue to increase as increasing amount of heparin was bound. There was less 'denaturation ' of bound heparin resulting in only a slight decrease in specific activity of heparin. This is in agreement with the hypothesis that the activation energy of the coupling reaction is high and hence at lower temperature it is less likely for a neighbouring tresylate to react with another amine on the bound heparin molecule. Figures 6-12 and 6-13 show graphically the change of total activity and specfic activity with time of the bound heparin respectively.

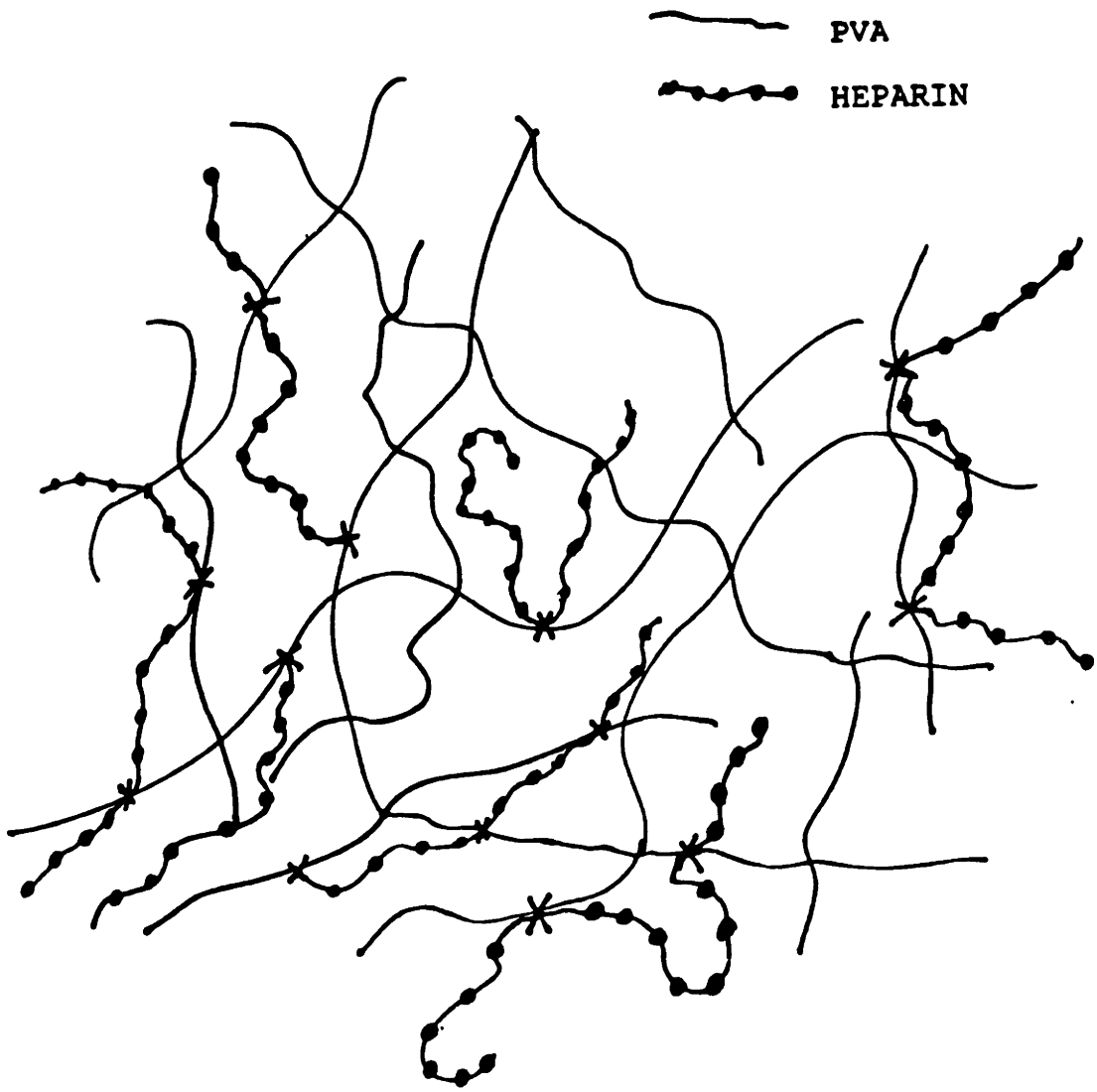


Figure 6-11 : Schematic diagram of the possible ways by which Heparin could be bound to tresylated PVA. An X is used to represent the point where a bond is formed between the two chains

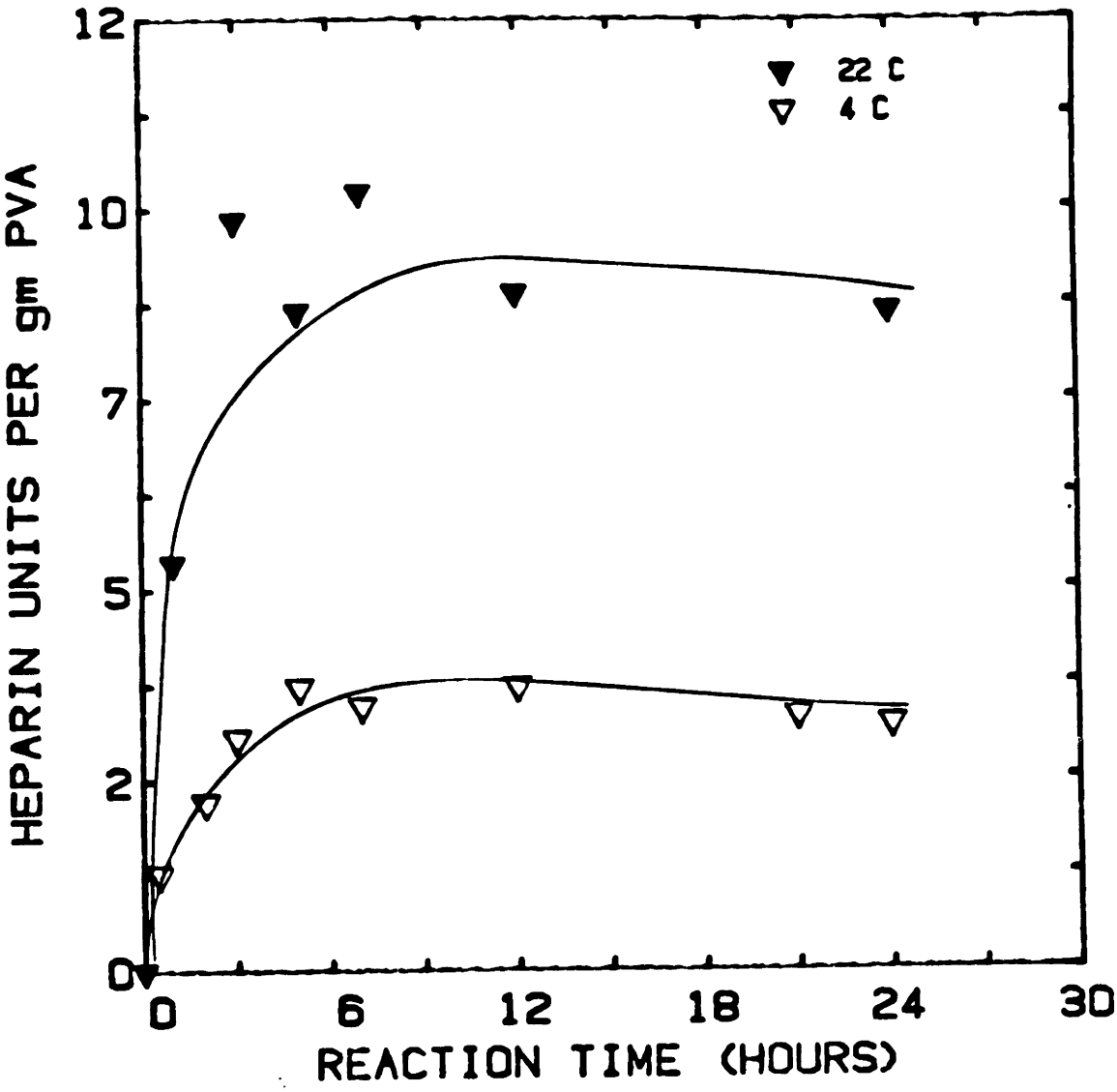


Figure 6-12 :Total Heparin activity found in PVA gel versus time of coupling.

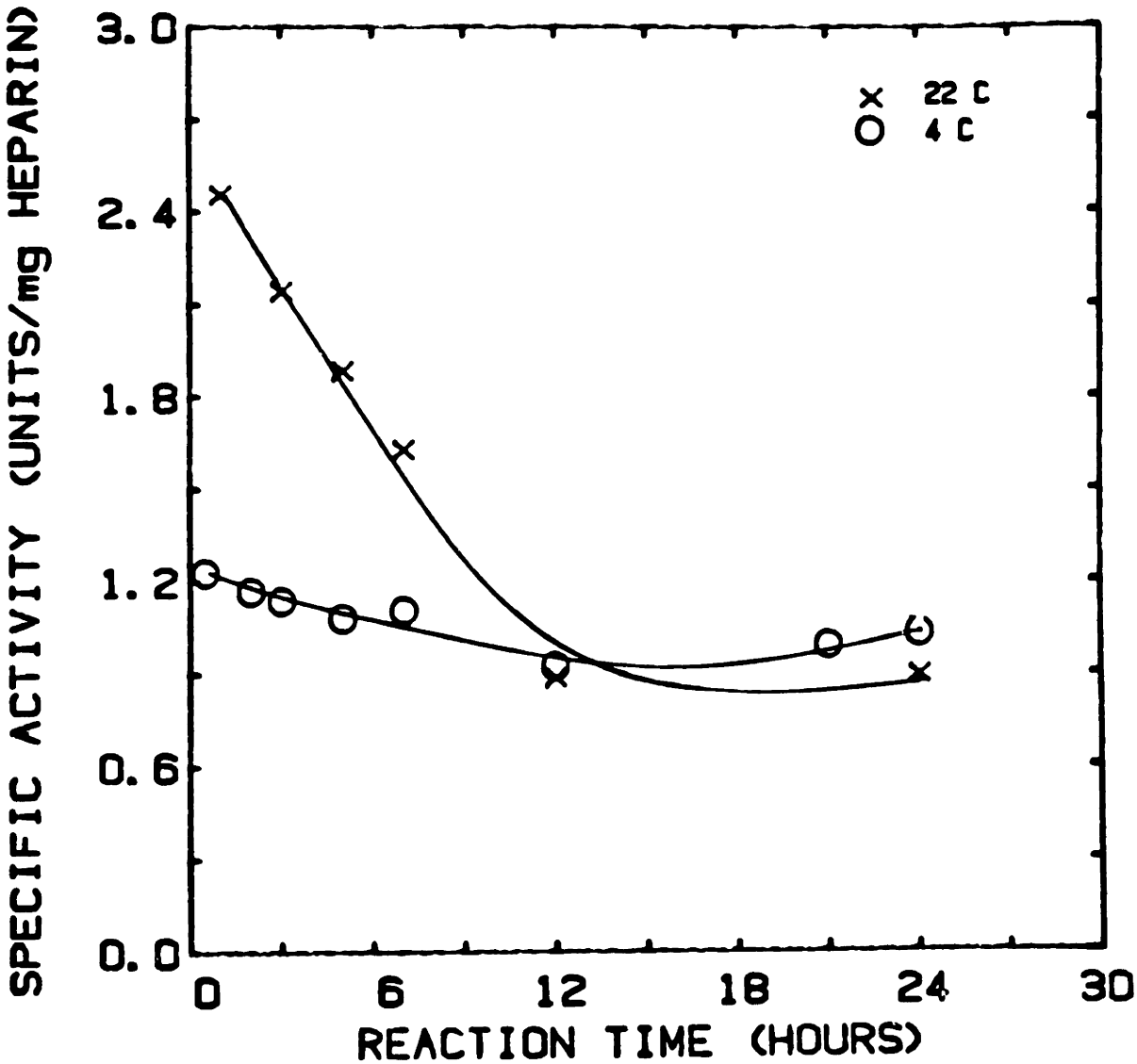


Figure 6-13 : Specific activity of the immobilised heparin on PVA with time of coupling

6.5 Effect of the Degree of Gel Activation on Immobilised Heparin

The various conditions used to prepare the samples in this study is given in table 6-8. The amount of tresylated hydroxyl as determined by the sulphur and fluorine content are also given. The ratios of fluorine and sulphur obtained in all cases are close to the theoretical value of 1.78

Figure 6-14 shows the amount of heparin bound as a function of activated group present on the PVA gel. As expected, the higher the activation achieved, the higher the amount of heparin coupled since in all instances an excess of heparin was used in the coupling. The specific activity of the bound heparin however decreased exponentially with the degree of activation. Higher activation led to greater denaturation of the bound heparin. The total amount of activity found in the gel is more or less constant for all the samples (table 6-9); the higher heparin content of the more activated gel compensating for the lower specific activity of the bound heparin.

The loss of activity of heparin when higher activated gel was due to the change in the proportion of heparin molecule bound at one position of the molecule only. Higher number of activated hydroxyls on PVA increases the probability of binding the heparin to PVA at multiple points leading to the loss of activity.

Table 6-8 : Degree of Activation of PVA with Tresyl Chloride in DMF at 22°C

Sample	TC added[a] (mM/gmPVA)	% F	% S	F/S	tresylate (uM/gmPVA)
TC20	0.453	0.047	0.028	2.04	8.75
TC40	0.906	0.082	0.051	1.61	15.9
TC60	1.359	0.16	0.092	1.739	30.25
TC80	1.812	0.28	0.15	1.84	46.8
TC100	2.265	0.30	0.18	1.714	56.2

Note: a - tresyl chloride added

Table 6-9 : Coupling of Heparin as a funtion of Degree of Activation with Tresyl Chloride at 22°C and pH 10

Sample	Heparin (mg/gmPVA)	Tot. Act. (U/gm PVA)	Specific Act. (U/mg hep)
TC20	0.667	9.2	13.79
TC40	0.97	6.75	6.96
TC60	3.31	6.56	1.98
TC80	4.33	7.9	1.82
TC100	9.3	8.3	0.89

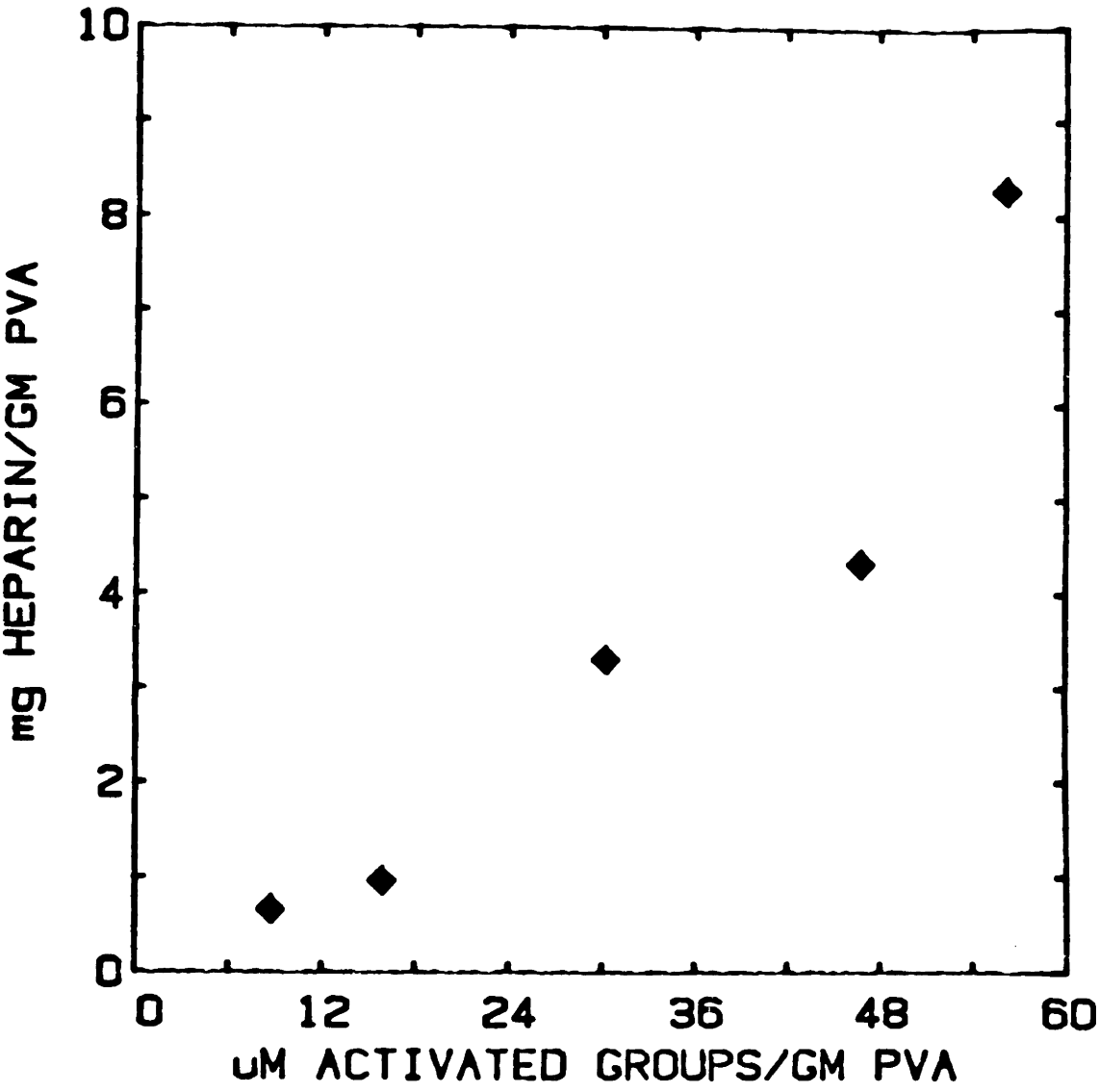


Figure 6-14 : Heparin bound as a function of Degree of Activation at 22°C and pH 10

6.6 Modified Heparin

Ideally the free amine group should only be located at one terminal of the heparin molecule so that it will be able to still assume a configuration similar to that found when in solution. The serine residue serves this purpose for a small percentage of the chains (14%) and of these some may also contain an unsubstituted glucoseamine which if attached at both points will result in a lost or decrease in activity. These glucoseamine can be quantitatively removed by controlled nitrous acid treatment cleaving the heparin at the position of the said glucoseamine with one of the fraction carrying an aldehyde.

The number of terminal amine groups can be increased by reductive amination of the reducing group in pure heparin and the nitrous acid heparin the results of which are elaborated in the next section.

6.6.1 Reductive Amination of Pure Heparin

PH was reductively aminated under two pH condition viz pH 7 and pH 10. Table 6-10 summarises the results of the amination. The success of the amination procedure was determined by the change in the number of reducing groups present in each preparation. Amination was more efficient at the higher pH. This is because amination is via the nucleophilic attack on the aldehydic carbon. Only the

unprotonated form of the amine is a strong nucleophile. At the higher pH of 10 the number of amine in the unprotonated

Table 6-10 : Reductive Amination of Pure Heparin

pH conditions	control	7	7	10
Reaction time(h)	-	24	72	24
Red. grp (uM/gm)	27.5	25.5	23.5	15
Serine (uM/gm)	22.2	22.2	22.2	22.2
Terminal amine(uM/gm)	22.2	24.2	26.2	34.7
% chains with terminal amines	14	15.2	15.9	21.9
% change in no. of terminal amines	-	9	18	56.3
% of red. terminal with amine	0	7.3	14.5	45.5

form is 3 orders of magnitude higher than at pH 7. The reaction scheme of the reductive amination is shown below:

Stable-ring <----------> Reactive aldehyde

heparin heparin

$$\text{A-CHO} + :\text{NH}_2\text{-B-NH}_2 \xrightarrow{\text{-------}} \text{A-CH=N-B-NH}_2 + \text{OH}^-$$

heparin hexadiamine Schiff's base

$$\text{A-CH=N-B-NH}_2 + 2[\text{H}] \xrightarrow{(\text{CNBH}_4)^-} \text{A-CH}_2\text{-NH-B-NH}_2$$

modified heparin

Cyanoborohydride will selectively reduce the Schiff's base and not the aldehyde or carboxylic acid at pH above 7.0 (Lane,1975). Therefore the decrease in reducing group after the above reaction is attributed to the attachment of a diamine to that group. After 24 hours at pH 10, about 45.5% of the available reducing group have been aminated (table 6-6). The rate determiining step in the amination is not the formation or reduction of the Schiff's base but rather the conversion of the cyclic form of the reducing sugar to the aldehye form. The cyclic form of the sugar is more stable and hence the majority of the sugars will be in that form and a very small percentage of the sugar are in the linear

form. Hexadiamine will react only with the linear aldehyde group.

Although there was a 56% increase in terminal amine after the amination reaction this amounts to only about 35 uM of terminal amine per gm or 22% of the chains now carry the terminal amine. The amination process did not result in a significant change in the activity of the heparin the activity of the PH10 being 151U/ml compared to 158U/ml in the original heparin.

Since the conversion of the ring form of the sugar to the linear form is the rate determining step, the reaction can be greatly accelerated if linear aldehydes were created at the chain terminal. One way of doing this is by subjecting the heparin to nitrous acid degradation.

6.6.2 Nitrous Acid Degraded Heparin

When heparin was treated with nitrous acid at pH 4, scission occured at the position where there was an unsubstituted glucoseamine. This resulted in a decrease in molecular weight.

Figure 6-15 is a plot of the molecular weight of the pure heparin before and after it has undergone the nitrous acid treatment. The degraded heparin still has a broad molecular weight distribution. The plot also shows that a small amount of the heparin in the higher molecular weight range is resistant to nitrous acid under these conditions indicating that there are some chains in the PH that do not

carry an unsubstituted glucoseamine and these chains are those that are in the higher molecular weight range.

Overall, there was a 35% decrease in M_n and a 100% increase in the number of reducing group generated. The

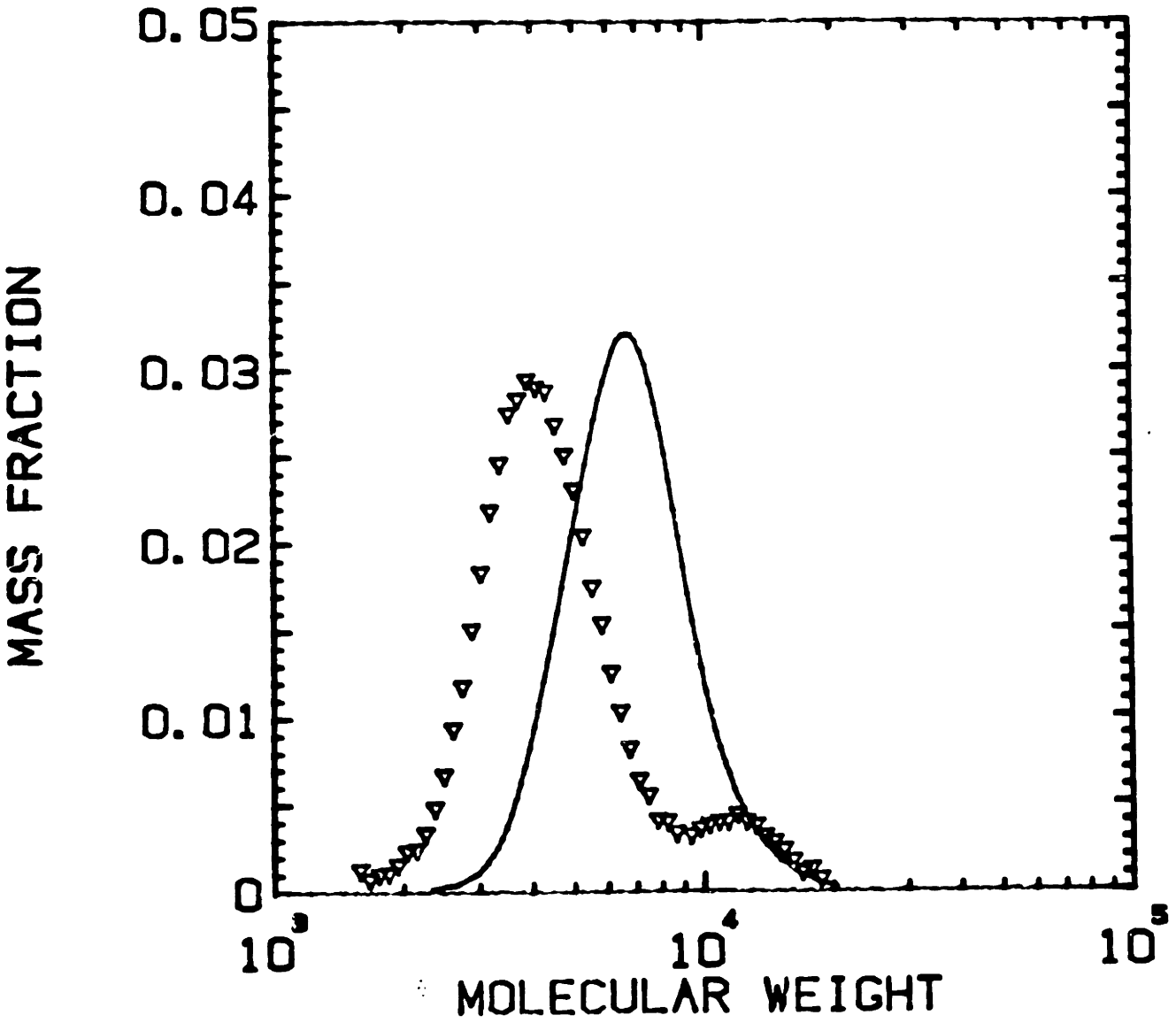
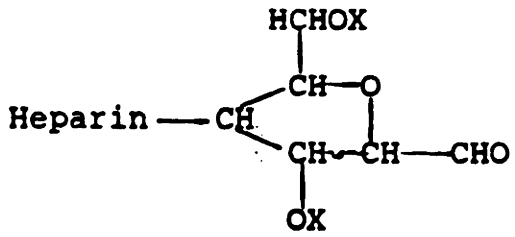


Figure 6-15 : Molecular weight distribution of Pure Heparin (——) and Nitrous Acid degraded Heparin (▽▽▽▽▽▽)

increase in reducing group was accompanied by approximately the same decrease in the number of primary amines in the sample as assayed by the fluorescamine assay. The loss of the primary amine group correspond very closely to the amine group attributed to glucoseamine in the original heparin indicating that degradation did indeed occur at that position and the oxidation of the glucoseamine is quantitative under the conditions used in this study.

The short reaction time of 10 minutes and the alkaline pH employed did not result in any significant N-desulphation of the 2-amino-2-deoxy-D-glucoside residue or a larger decrease in molecular weight will be observed.

The degradation resulted in the generation of a aldehyde species at the reducing end of only one of the two chain generated by the degradation. This species is anhydromannose and it carries a reactive linear aldehyde at its C_1 atom.



Anhydromannose

where OX = acetate, sulphate or hydroxyl group

The reaction of this group with hexadiamine is quantitative and facile. A high excess of diamine was used to prevent the former from bridging two molecule. The SEC plot of the MHNO and MHNH are shown in figure 6-16. The two samples have identical molecular weight. Amination did not result in significant increase in molecular weight.

The results from the amination and nitrous acid degradation are summarised in table 6-12. The amine measured in MHNH sample is contributed by the serine residue and the hexadiamine bound to the reducing terminals. The procedure used to remove excess diamine was successful in removing all traces of unreacted diamine. Figure 6-17 is a photograph of the results of the high voltage paper electrophoresis. Traces of hexadiamine found in the eluent from the sephadex-G25 column was removed completely after passage through the cation exchanger as seen by the absence of the ninhydrin colour complex of lane 3 in figure 6-17.

From the reducing group and primary amine content of the aminated heparin, it is clear that all the reducing functions generated by the nitrous acid treatment were successfully coupled to a diamine. About 48% of the original reducing terminal were also converted to carry a terminal amine. This results in a 171% increase compared to 45% in PH10 in the number of chain with a terminal amine although

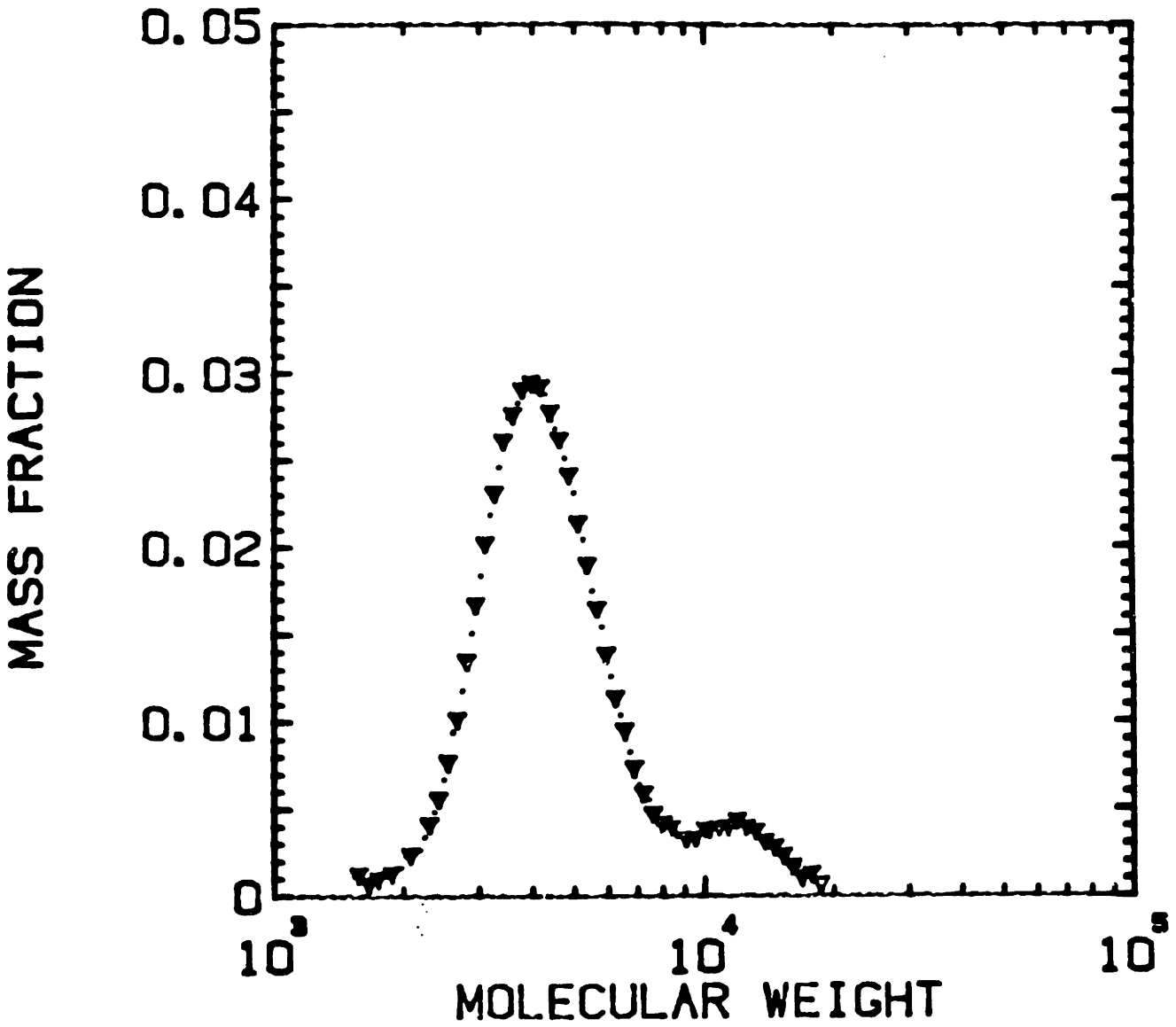


Figure 6-16 : Molecular Weight Distribution of Nitrous acid degraded heparin before (····) and after (▼▼) reacting with Hexadiamine.

Table 6-12: Effect of Nitrous Acid degradation and Reductive Amination on the Reducing group and primary amine content of Heparin

	PH	MHNO	MHNH
M_w	7000	5045	5045
M_n	6300	4100	4100
Red. group(uM/gm)	27.5	58.8	14.2
% change in red.grp	-	113.8	-48.4
Total 1^o amine(uM/gm)	56.7[a]	18.2[a]	60.3[b]
Serine (uM/gm)[c]	22.2	22.2	22.2
$GluNH_2$/hexadiamine (uM/gm)[d]	34.5	0	38.1
Terminal amine (uM/gm)	22.2	22.2	60.3
% Molecule with terminal amine	14	9.1	24.7

a - based on glucose amine
b - based on hexadiamine
c - assumed that modification process did not change affect the serine residue
d - obtained by subtracting the contribution due to serine from the total amine content. For MHNH the amine is mainly from hexadiamine while for PH it is only from glucoseamine

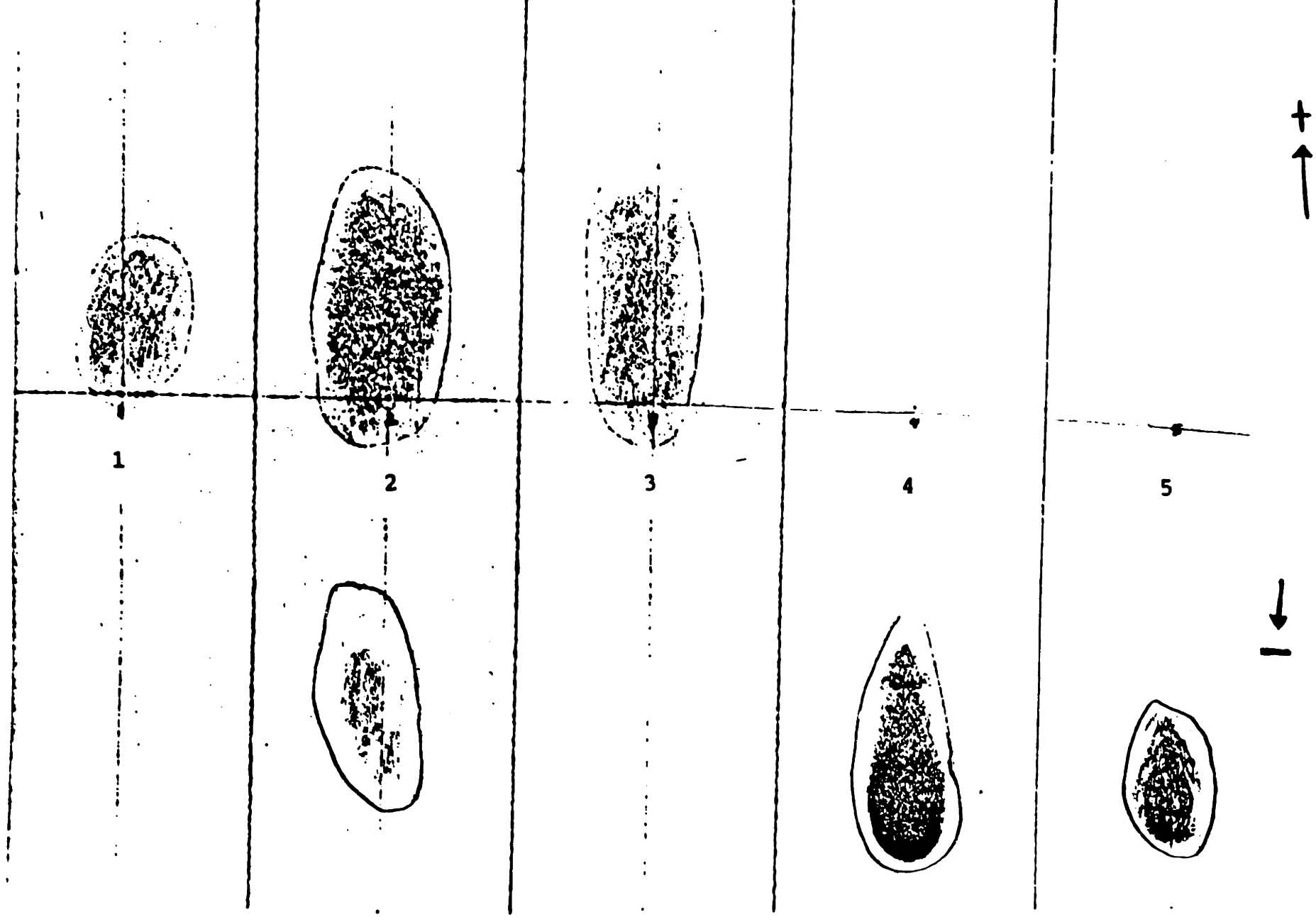


Figure 6-17 : High Voltage Paper Electrophoresis of Heparin and Hexadiamine. pH 8.5, 1700V. Lane 1 - Pure Heparin; Lane 2 -Heparin - Hexadi amine mixture purified twice on the G-25 sephadex column; Lane 3 - heparin/hexadiamine mixture purified twice on G-25 column and once through the cation exchanger. Lane 4 - pure hexadiamine and Lane 5 - heparin/hexadiamine mixture

only 25% of the chain present carried the terminal amine.

The activity of the heparin after undergoing the nitrous acid treatment and amination was found to be 115U/mg compared to 158U/mg in the original heparin. This constitute a loss of 27% in activity in free solution. However the increase in the terminal amines and the absence of glucoseamines along the backbone resulted in an increase retention of activity after this heparin was bound as shown below. There is also some evidence that shorter chain heparin is less likely to cause thrombotic complications like thrombocytopenia and embolism (Lasker and Chui,1973; Hirsh et al. 1985).

The above results showed that the nitrous acid degraded heparin will most likely contain only terminal amines and no unsubstituted glucoseamine. Therefore binding of this heparin to a substrate via an amine nucleophilic attack will result in only one type of configuration i.e. end terminal linked heparin.

6.6.3 Coupling of Modified Heparin onto PVA

Modified heparin (MHNH and PH10) were coupled to PVA at the lowest activation studied in SECTION 6.5 since the above results indicate the highest specific activity found at this level of activation. Table 6-13 compares the specific activity of the three types of heparin when bound to TC20 gels. The amount of heparin bound in all three cases are approximately the same for all three cases.

Table 6-13 : Modified Heparin bound to PVA

	PH	PH10	MHNH
mg Hep/gm PVA	0.667	0.36	0.44
Units/gm PVA	9.2	1.0	17.0
Sp. Act. U/mg Hep	13.8	2.78	38.6
% retention of Act.[a]	8.72	1.76	38.63

a- retention of activity based on 158U/mg for PH and PH10 and 115U/mg for MHNH

MHNH which have undergone nitrous acid degradation first have the highest specific activity (38.6 U/mg) which is 180% higher than when pure heparin was used. PH10 on the other hand has a lower activity than that of PH. The removal of unsubstituted glucoseamine by nitrous acid degradation and replacement with a hexadiamine residue at the reducing ends effectively prevent the heparin from being coupled to the PVA at multiple sites. Figure 6-18 is a schematic diagram of how the MHNH heparin will be most likely bound to heparin. Almost all of the bound heparin will be linked via the amino terminal and this configuration was postulated to be able to retain the highest amount of activity. Attachment of the hexadiamine also adds a spacer of about 6 carbon chain between PVA and the heparin molecule allowing the molecule to protrude more into the solvent and thus decreasing the steric restriction of the support and from that of structured water micro environment. This facilitates correct orientation of the heparin and enhances the affinity between heparin and the proteins (O'Cara,1978).

The activity of PH10 was lower than that of pure heparin even though the number of terminal amine increased 56%. It implies that having heparin with a high serine content and thus more terminal amine is a necessary but not the only criteria needed to ensure active bound heparin when

a substrate like PVA is used where the hydroxyls are found all along the main chain. Increasing the terminal amines

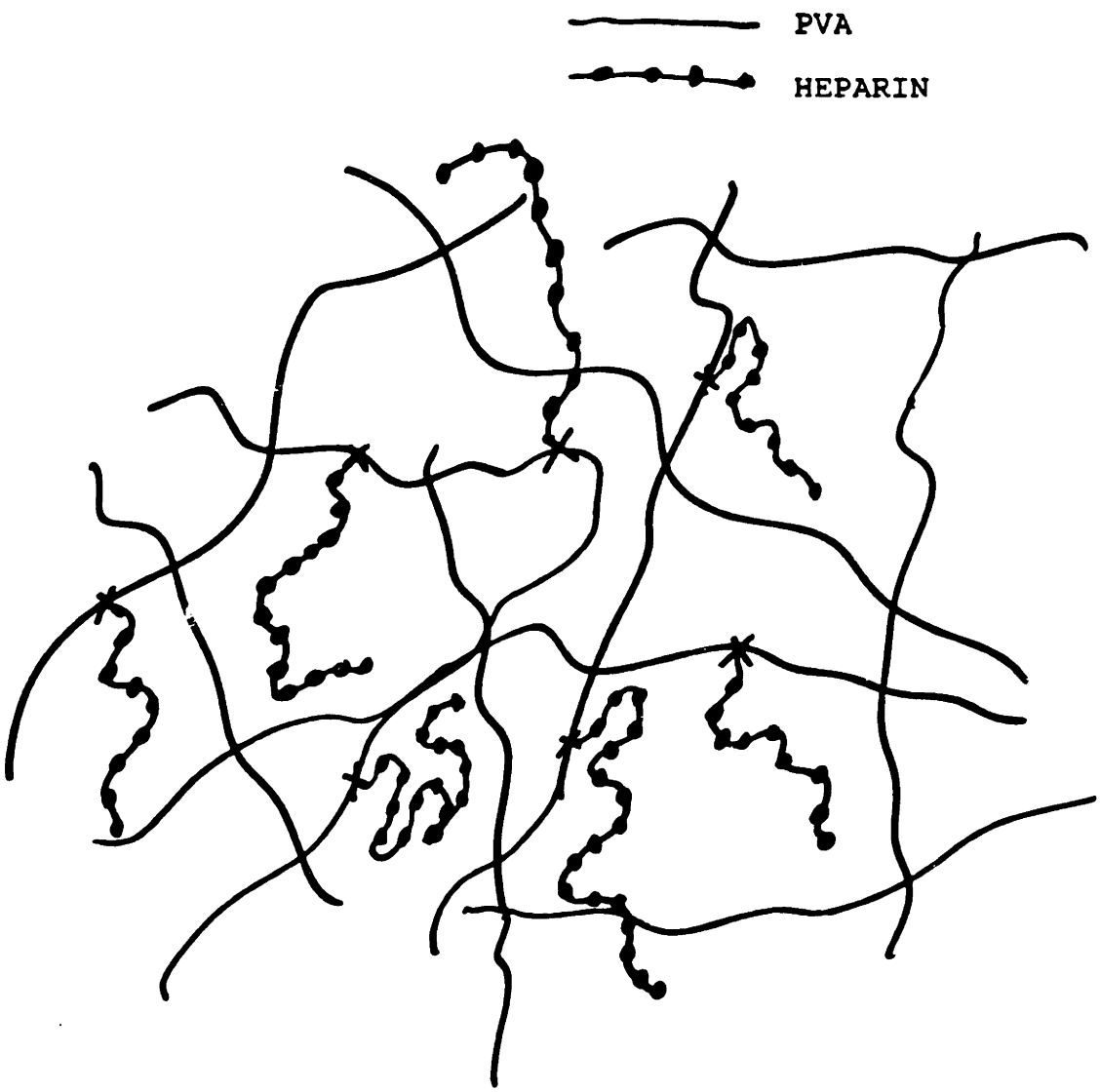


Figure 6-18: Schematic diagram of the binding of nitrous acid modified heparin onto tresylated PVA. At the position where a bond is formed between the two molecule an X is drawn

without changing the distribution of tresylates along PVA and the free amine along the backbone probably increased the incidences of heparin being bound at more than one site.

6.7 Coupling of Pure Heparin to PEO

Another way of improving the activity of the bound heparin without subjecting the heparin to nitrous acid degradation and then amination is to space out the tresylate found on the substrate. This would limit the reaction of the tresylate to only one amine on the heparin molecule.

$$HO\text{-}(\text{-}CH_2\text{-}CH_2\text{-}O\text{-})_n\text{-}H$$

Poly(ethylene oxide)

Poly(ethylene oxide) whose general formula is given above contains only terminal hydroxyls at either ends of the polymer. These hydroxyls are primary hydroxyls which are more reactive than the secondary hydroxyls found in PVA which will compensate somewhat for the lower number to which the heparin can be coupled. The hydroxyls are separated from each other by its $\langle r^2 \rangle^{1/2}$ although the spatial distances of these hydroxyls will be slightly different depending on the polymer fraction of the gel. When activated with tresylate the latter will likewise be separated by the same distances. Coupling of heparin to these polymer will produce

gels with much greater activity than those obtained with PVA even at high activation and coupling if the above postulates are correct. This was indeed found to be the case as shown by the specific activities of the two type of gels as shown in Table 6-14 which compares the activity of the gel when pure heparin was bound to PVA and PEO. The total amount of heparin bound was comparable in both cases. 10.3% of initial activity was retained when PEO was used compared to 1.15% when heparin was bound to PVA for reasons given above. Moreover the PEO chain to which the heparin was attached acts as a leach the length of which depends on the crosslink density of the network. This reduces the steric hindrance of the network and contribute to the higher retention of heparin activity. Figure 6-19 is a schematic diagram of heparin bound to PEO.

Table 6-14 : Binding of Heparin to PVA versus PEO Hydrogel

	PVA	PEO
mg hep/gm polymer	4.33	5.83
Units/gm polymer	7.9	95.0
Specific Act. (U/mg Hep)	1.82	16.3
% Retention of Activity	1.15	10.3

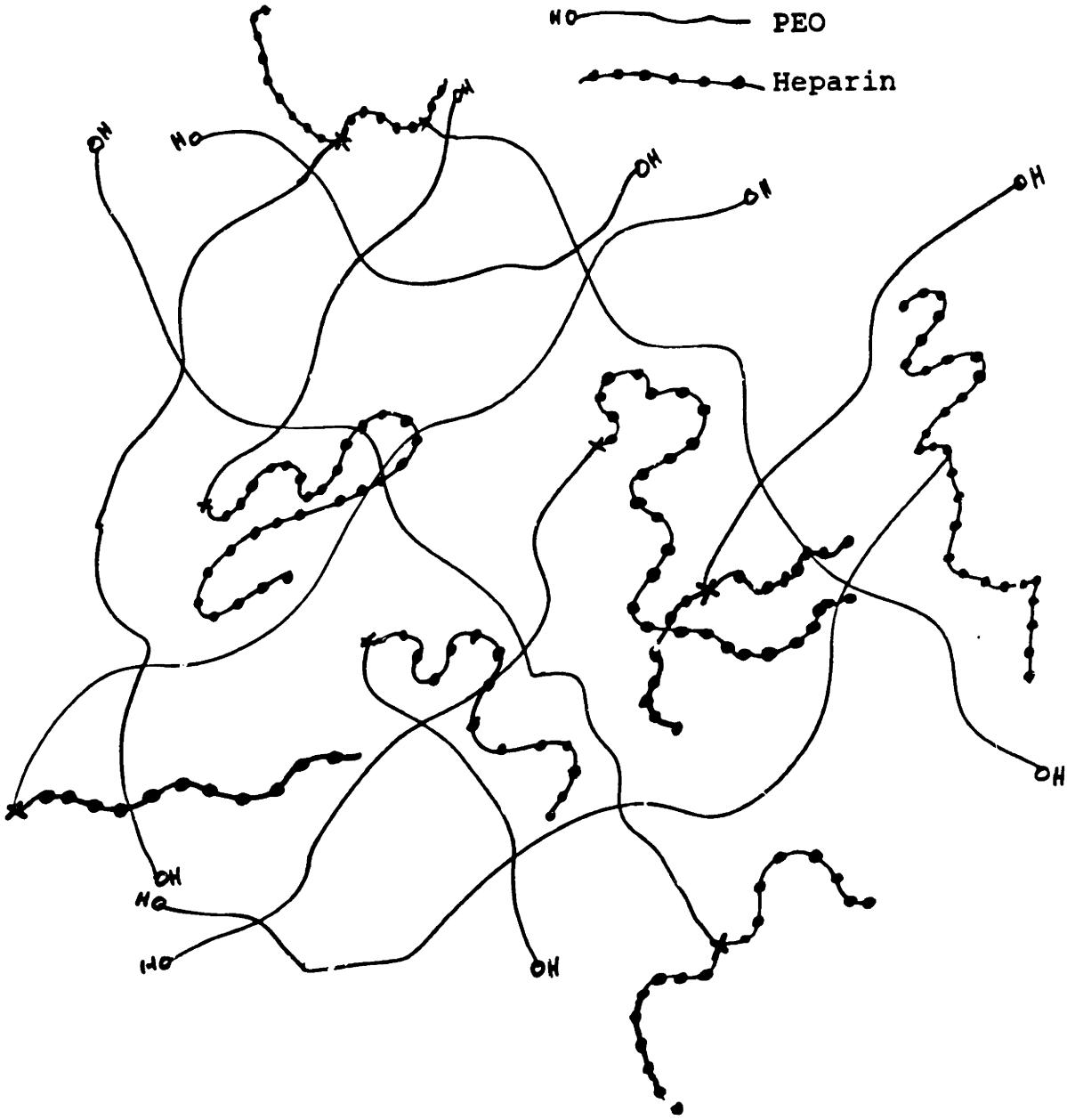


Figure 6-19 : Schematic diagram of heparin bound to PEO network. An X is used to represent a bond formed between the Heparin and PEO network

7 CONCLUSION

Heparin bound to hydroxyl containing polymers via Tresyl Chlorides produced an imine bond which is stable to hydrolysis and enzyme degradation in plasma.

Higher temperature of coupling of heparin to PVA increased the amount of heparin bound but not its activity due to the increase of heparin molecule that were bound to the substrate at more than one point along the molecule.

The factors affecting the activity of heparin bound to the hydrogel were:

1. the manner in which heparin was bound to the hydrogel - specifically whether at its terminals or along its chain
2. the number of links between a single heparin molecule and the substrate
3. the length of the leash to which the heparin was attached

The highest activity of immobilised heparin was obtained:

1. By using lightly tresylated PVA with the unmodified commercial heparin: this is not a very satisfactory method as it limits the total amount of heparin that can be bound.

2. Removal of glucoseamine along the heparin backbone by nitrous acid degradation and attaching a diamine at the terminal to

produce molecules with terminal amines only via which the heparin can be attached to the matrix. This produces shorter heparin but the retention of activity was 300% higher than when unmodified heparin was used even at low activation. The modification also introduced a leash of 6 carbon between the heparin and the hydrogel.

3. By using a polymer that has functional group spaced apart with the commercial heparin to prevent the binding of heparin at more than one point. The functional group should ideally be separated by a distance larger than the radius of gyration of the heparin molecule. Heparin bound to PEO was about 10 times more active than PVA. Its lower hydroxyl content is compensated by the higher reactivity of the primary hydroxyls.

9. REFERENCES

Abe,K. (1959). "Irradiation of PVA with heating." Nippan Hoshasen Kenkyu Kyokai Nempo, vol. 1.

Ansell, J.; Slepchuk, N;, Kumar, R; Lopez, a.; Southard, L. and Deykin, d. (1986). "Heparin-induced thrombocytopenia: A prospective study." Throm. and Haemost. 43(1), pp. 61-65.

Baier, R.E.; Shafrin, E.G. and Zisman, W.A. (1968). "Adhesion:Mechanism that assist or impede it." Science, vol. 162, pp. 1360

Bauer,K. (1972) "Radiation Crosslinked PVA Solution as model biomaterials," S.M. Thesis, Dept. of Chem Eng. MIT Cambridgem MA 02139

Berger, S.; Salzman, E.W. (1974). "Thromboembolic complication ofprosthetic devices." In Spaet. 7th ed.:Progress in Hemostasis and Thrombosis, Vol. 2, Grune and Stratton, New York, pp. 273-309.

Berkowitch, J; Charlesby, A. and Desreux, V. (1957). "Radiation effects on aqueous solution of PVA." J. Poly. Sci., vol. 25, pp.490.

Bethel, G.S.; Ayers, J.S.; Hancock, W.S. and Hearn, M.T.W. (1979). A novel method of activation of cross-linked agarose with 1,1'-carbonyldiimidazole which gives a matrix for affinity chromatography devoid of additional charge groups. J. of Biol. Chem., vol. 254 (8), pp. 2572-2574.

Bethel, G.S.; Ayers, J.S.; Hearn, M.T.W. and Hancock, W.S. (1981a). Investigation of the activation of various insoluble polysaccharides with 1,1'-carbonyldiimidazole and of the properties of the activated matrices. J. of Chrom., vol. 219, pp. 361-371.

Bethel, G.S.; Ayers, J.S.; Hearn, M.T.W. and Hancock, W.S. (1981b). Investigation of the activation of cross-linked agarose with carbonylating reagents and the preparation of matrices for affinity chromatography purifications. J. of Chrom., vol. 219, pp. 353-359.

Bovey, F.A. (1958). "The effects of ionising radiation of natural and synthetic high polymers." Interscience, New York.

Brandrup, J. and Immergut, E.H. (editors). (1979). Polymer Handbook, Second Edition, John Wiley.

Bray, J.C. (1972). "Formation of polyvinyl alcohol hydrogels via electron beam irradiation and crystallization -- application as asynthetic articular cartilage material." Sc.D. Thesis, M.I.T.,Cambridge, Massachusetts.

Castellot, J.J. Jr.; Favreau, L.V.; Karnovsky, M.J. and Rosenberg, R.D. (1982). "Inhibition of vascular amooth muscle cell growth by endothelial cell-derived heparin. Possible role of a platelet endoglycosidase." J. Biol. Chem. 257(19), pp. 11256-60.

Chapiro, A. (1962). "Radiation chemistry of polymeric system."Interscience, New York.

Cholakis, C.H. and Sefton, M.V. (1983). "Chemical characterization of an immobilised heparin-PVA." Polymer Preprints.

Cifonelli, J.A. (1968). Carbohydrate Research, vol. 8, pp. 233.

Coates, R.M. and Chen, J.P. (1969). Tetrahedron Loit, pp. 2705.

Crossland R.K.; Wells, W.E. and Shiner V.J. Jr.(1971) "Sulphonate Leaving groups, Structure and Reactivity." J. Amer. Chem. Soc. vol. 93(17) pp 4217-4219

Crossland, R.K. and Servis, K.(1970) "A facile synthesis of methanesulphonate ester.
Cuatrecasas, P.J. (1970). J. Biol. Chem., vol. 245, pp. 3059.

Danno, A. (1958a). "Effects of ionising radiation on PVA. I.Breakdown of main chain and formation of carbonyl groups." J.Phy. Soc., Japan, vol. 13, pp. 609.

Danno, A. (1958b). "Gel formation of aqueous solutions of PVA by gamma-rays from Co-60." J. Phy. Soc., Japan, vol. 13, pp. 722.

Dennison, K.A.(1986) "Radiation crosslinked PEO hydrogel membrane" Ph. D. Thesis, MIT, Cambridge, MA 02139

Dincer, A.K. (1977). "Covalent coupling of heparin to synthetic polymer surfaces." Ph.D. Thesis, M.I.T., Cambridge,Massachusetts.

Ebert, C.D.; Lee, E.S.; Deneris, J. and Kim, S.W. (1982). "The anticoagulant activity of serialised and immobilised heparin."Adv. in Chem. Series, #199, A.C.S., Washington D.C.

Engelberg, H. (1963). Heparin: Metabolism, Physiology and Clinical Applications. Charles C. Thomas, Springfield, Illinois.

Finch, C.A. (1981). "Some properties of polyvinyl alcohol and their possible applications." In: C.A. Finch (ed.), Chemistry and Technology of Water Soluble Polymers, Plenum Press, New York.

Fougnot, C.; Labarne, D; Jozcfonvicz, J.; Fozefovicz, M. (1984). In: G.W. Hastings and P. Ducheyne (ed.), Macromolecular Biomaterials, chapter 9.

Fujiwara, S; Fujiwara, W; Fujii, K. and Fukoroi, T. (1966). J.Mol. Spect., vol. 19, pp. 294.

Gorden, J.L. and Milner, A.J. (1976) " Blood Platelets as multifunctional cells" Platelets in Biology and Pathology vol 1, pp.3-32; J.L. Gorden ed. N. Holland Publ. Co. NY

Gott, V.T.; Wiffen, J.D. and Dutton, R.C. (1963). "Heparin bonding on colloidal graphite surfaces." Science, col. 142, pp.1297

Griffin, M. (1981). "The significance of the interaction of heparin with thrombin pertaining to the heparin enhanced-antithrombin III/thrombin reaction time." In: R.L.Lundblad, W.V. Brown, K.G. Mann and H.R. Roberts (ed.),Chemistry and Biology of Heparin, Elsevier North Holland, Inc.

Griffin, M.J. (1983). "Heparin-catalysed inhibitor/protease reactions: kinetic evidence for a common mechanism of action of heparin." Proc. Natl. Acad. Sci. USA., 80(18), pp. 54606-4.

Grode, G.; Falb, R. and Anderson, S. (1969). Artificial Heart Program Conference, US Dept. of Health, Education and Welfare,NIH, Washington D.C., pp. 19.

Halpern, B.D. and Shirakawa. (1968). Adv. Chem. vol. 87,pp. 197.

Hearn, M.T.W.; Bethel, G.S.; Ayers, J.S. and Hancock, W.S. (1979). Application of 1,1'-carbonyldiimidazole-activated agarose for the purification of proteins. II. The use of an activated matrix devoid of additional charged groups for the purification of thyroid proteins. J. of Chrom., vol. 185, pp. 463-470.

Hirsh, J.; Ofosu, F. and Buchanan, M. (1985). "Rationale behind the development of low molecular weight heparin derivatives." Semin. Thromb. Hemost. 11(1), pp. 13-6.

Hiruno, Y. (1964). "Radiation induced main chain scission in PVA." Bull. Chem. Soc., Japan. vol. 37, pp. 1945.

Hoffman, A.S.; Schmar, G.; Harris, C. and Kraft, W.G. (1972).Trans. Am. Soc. Artif. Int. Organs, vol. 18, pp. 16.

Holmer, E.; Mattsson, C. and Nilson, S. (1982). "Anticoagulant and antithrombotic effects of heparin and low molecular weight heparin fragments in rabbit." Thromb. Res., vol.25(6), pp.475-483.

Holmer, E.; Soderstrom, G. and Andoson, L.O. (1979). Eur. J. Biochem. vol. 93, pp. 1-5.

Horton,D. and Philips, K.D. (1973) Carbohydr. Res. vol 30, pp. 367

Hrushesky, W.J. (1978). "Subcutaneous heparin induced thrombocytopenia." Arch. Intern. Naed. 138(10), pp. 1489-91.

Jaques, L.B. and Wollin, A. (1967). "A modified method for the colorimetric determination of heparin." Can. J. of Physiol. and Pharmcol. vol.45, pp. 787-794.

Jeanloz, R.W. (1975). "Heparin: structure, function, and clinical implications." In: R.A. Bradshaw and S. Wessler (ed.),The Chemistry of Heparin, Plenum Press, New York, pp. 3-17.

Jordan, R.; Deeler, D. and Rosenberg, R. (1979). "Fractionation of low molecular weight heparin species and their interaction with AT3." J. Biol. Chem. 254(8), pp. 2903-13.

Jordan, R.E.; Oosta, G.M.; Gardner, W.T. and Rosenberg, R.D. (1980). J. Biol. Chem. vol. 225, pp. 10081-90.

Karapally, J.C. and Dietrich, C.P. (1970). "A uronic acid isomerase in Flavobacterium heparinium." Can. J. Biochem., vol.48, pp. 164.

Karp, G.I., Marcum, J. and Rosenberg, R. (1984). "The role of tryptophan residues in heparin-antithrombin interaction." Archives of Biochem. & Biophysics. vol. 233(2), pp. 712-720.

Klenin, V.I.; Kletrina, O.V. and Galaktionov, V.U. (1966). "Study of supermolecular particles in aqueous solution of PVA." Vysokomal. Soyed., Translated in Polymer Sci., USSR., vol. 8(11), pp. 2192.

Knight, L.C.; Budzynski, A.Z. and Olexa, S.A. (1981)

"Radiolabelling of fibrinogen using the Iodogen Technique." Thromb. Haemostas. vol 46(3), pp 593-596.

Kohn, J. and Wilchek, M. (1978). Biochem. Biophysics. Res. Commun. vol. 84, pp. 7-14.

Lagunoff, D. and Warren, G. (1962). Arch. Biochem. Biophys., vol. 99, pp. 396.

Lane, C.F. (1975). "Sodium cyanoborohydride - a highly selective reducing agent for organic functional groups." Synthesis, March 1975.

Langer, R.: Linhardt, R.J.; Galliher, P.M.; Flanagan, M.M.; Gooney, C.L. and Klein, M.D. (1982). "A system for heparin removal." In: S.L. Cooper, and N.A. Poppas (ed.), Biomaterials: Interfacial Phenomena and Application, Advances in Chemistry Series, #199, ACS, Washington, D.C.

Larsson, R.; Eriksson, J.C.; Lagergren, H. and Olsson, P. (1979). "Platelet and plasma coagulation compatibility of heparinised and sulphated surface." Throm. Res. vol. 15, pp. 157-167.

Lasker,S. and Chui,M. (1973). "Orally active Anticoagulant" US Patent 3,766,167, 1973

Laurent, T.C.; Tengblad, A.; Thernberg, L.; Hook, M. and Lindahl, U. (1978). Biochem. J. vol. 175, pp. 691-701.

Lindahl, A.; Bockstrom, G.; Jansson, L. and Hallen, A. (1973). J. Biol. Chem. vol. 249, pp. 7234.

Lindahl, U.; Thunberg, L.; Backtrom, G. and Riesenfeld, J.(1981). "The antithrombin-binding sequence of heparin."Biochem. Soc. Trans., vol. 9, pp. 499-501.

Lindon, J.; Rosenberg, R.; Merril, E. and Salzman, E. (1978). "Interaction of human platelets with heparinised agarose gel." J. Lab. Clin. Med. vol 91(1), pp. 47-49.

Lindon, J.N.; Salzman, E.W.; Merrill, E.W.; Dincer, A.K.; Labarre, D.; Bauer, K.A. and Rosenberg, R.R. (1985). "Catalytic activity and platelet reactivity of heparin covalently bonded to surfaces." J. Lab. Clin. Med. vol. 105(2). pp. 219-26.

Linker A. and Hovingh P. (1972) "Purification of Heparinase from A. heparinium" Methods in Enzymology vol XXVIII V. Ginsberg ed. pp 902 Publ Acad. Press.

Lorand, J.P. and Edwards, J.O. (1959). "Polyol complexes and structure of the benzene boronate ion." J. Org. Chem., vol.24,pp. 769-774.

Merrill, E.W. and Salzman, E.W. (1983). "PEO as a biomaterial."ASAIO, April-June, vol. 6(2), pp. 60-64.

Merrill, E.W.; Salzman, E.W. and Wong, P.S.L. (1970). "Polyvinylalcohol-heparin hydrogel 'G'." J. Appl. Physiol., vol. 29, pp.723.

Morrison, R.T. and Boyd, R.N. (1969). Organic Chemistry, Second Ed., Allyn and Bacon Inc., Boston, USA, pp. 532.

Matsuo, T. and Inagaki, H. (1967). "Uber den losungszusta and des polyvinyl alkohols in wasser." Makromol. Chem., 53, pp. 130.

Nilsson, K. and Mosbach, K. (1980). "p-Toluene sulphonyl chloride as an activating agent of agarose for the preparation of immobilised affinity ligands and proteins." Eur. J. Biochem., vol. 112, pp. 397-402.

Nilsson, K. and Mosbach, K. (1981). "Immobilization of enzymes and affinity ligands to various hydroxyl group carrying supports using highly reactive sulphonyl chlorides." Biochemical and Biophysical Res. Comm. vol. 102(1), pp. 449-457.

Nilsson, K. and Mosbach, K. (1984). "Immobilisation of ligands with organic sulphonyl chlorides." In: W.B. Jacoby (ed.) Methods in Enzymology, Academic Press, vol 104(part C), pp. 57-69.

Nordenman, B.; Nystrom, C. and Bjork, I. (1977). Eur. J. Biochem. vol.78, pp. 195-203.

Nordenman, B.; Bjork, I. (1978). "Binding of low-affinity and high-affinity heparin to AT3. UV difference spectrography and circular dichroism studies." Biochemistry, vol. 17(16), pp. 3339-44.

O'Carra, P. (1978). In: R. Epton (ed.), Chromatography of Synthetic and Biological Polymers, Vol. 2, Hydrophobic, Ion Excluding and Affinity Methods, Ellis Horwood, Chichester, England, pp. 131.

O'Donnel, J.H. and Sangster, D.P. (1970). Principles of Radiation Chemistry. Edward Arnold Publishers Ltd., London, pp.52.

Oldberg, A.; Helden, C.H.; Wasteson, A.; Busch, C. and Hook, M. (1980). "Characterisation of a platelet endoglycosidase

degrading heparin-like polysaccharides." Biochemistry, vol. 19(25), pp. 5755-62.

Oosta, G.M.; Favreau, L.V.; Beeler, D.L. and Rosenberg, R.D. (1982). "Purification and properties of human platelet heparitinase." J. Biol. Chem. vol. 257(19), pp. 11249-55.

Olsson, P.; Arnander, C.; Larm, O. and Larsson, R. (1983). "Preparation of a thromboresistant surface by selective covalent binding of heparin via a modified reducing terminal residue." Thrombosis and Hemostasis, vol.50(1), pp. 22.

Pekala, R.W. (1984) "Synthesis and Characterisation of Polyether/Polysiloxane networks for Blood-Interfacing Applications" Ph. D. thesis. Dept of Mat. Sci. and Eng. MIT Cambridge Massachusetts 02139

Peppas, N.A. (1973). "Crystalline radiation cross-linked hydrogel of PVA as potential biomaterials." Ph.D. Thesis,M.I.T., Cambridge, Massachusetts.

Plato, N.A.; Valuer, L.I. and Gumirova, F.K. (1979).Preprints, Makromainz, vol. 3, pp. 1522.

Pletcher, C.H. and Nelsestuen, G.L. (1982). "The rate determining step of the heparin-catalysed antithrombin/thrombin reaction is independent of thrombin." J. Biol. Chem. vol. 257(10), pp. 5342-5.

Pritchard, J.G. (1970). Polyvinyl Alcohol: Basic Properties and Uses. Gorden Breach Publishers, New York.

Rosenberg, R.D. (1982). "Heparin-antithrombim system." In:R.W. Coleman, J. Hirsh, V.J. Marder and E.W. Salzman (ed.),Hemostasis and Thrombosis: Basic Principles and Clinical Practice, J.B. Lippincott Co., pp. 962-985.

Rosenberg, R.D. and Lam, L. (1979) "Correlation berween structure and function of heparin" Proc. Natl. Acad. Sci. USA, vol 76(3), pp1281.

Saba, H.I.; Saba, S.R. and Morelli, C.H. (1984). "Effect of heparin on platelet aggregation." Am. J. Hematol. vol. 17(3), pp. 295-306.

Sakurada, I. and Ikada, Y. (1961). "Effects of cobolt-60 gamma-radiation on PVA." Bull. Inst. Chem. Res., Kyoto University, vol. 39, pp. 99.

Sakurada, I.; Nakajima, A. and Fujiwara, H. (1959). "Vapor pressure of polymer solutions. II. Vapor pressure of PVA-water system." J. Poly. Sci., vol. 35, pp. 497-505.

Sakurada, I.; Okada, H. and Kimira, H. (1959). "Gel formation by irradiation of PVA with heating." Nippon Hoshasen Kenykyu Kyokai Nempo, vol. 1, pp. 119-120.

Salzman, E.W. and Merrill, E.W. (1982). "Interaction of blood with artificial surfaces." In: R.W. Colman, J. Hirsh, V.J.Marder and E.W. Salzman (ed.), Hemostasis and Thrombosis: Basic Principles and Clinical Practice, J.B. Lippincott Co., pp.931-943.

Salzman,E.W.; Merrill,E.W.; Binder, A.; Wolf, C.F.W.; Ashford,T.P. and Austen,W.G. (1969). "Protein-platelet interaction on heparinised surfaces." J. Biomed. Mater. Res., vol.3, pp.69-81.

Schnabel, W. (1967). "Simultaneous crosslinking and degradation of polymers in organic solvent by gamma-rays." In: J. Dobo and P. Hedvig (ed.), Proc. of the Second Tihany Symp. on Rad. Chem.,Akademia Kiado, Budapest.

Scouten, W.H. and Dertweel, W.V. (1984). "Chromophoric sulphonyl chloride agarose for immobilising bioligands." Annals of NY Acad. Sci., vol. 434(Dec), pp. 249-250.

Sefton, M. and Goosen, M.F.A. (1981). "Irreversible immobilization of heparin for biomaterials." In: P.L. Lundblad,W.V. Brown, K.G. Mann and H.R. Roberts (ed.), Chemistry and Biology of Heparin, Elsevier North Holland Inc.

Shively, J.E. and Conrad, H.E. (1970). Biochem., vol. 9, pp. 33.

Shively, J.E. and Conrad, H.E. (1976). "Formation of anhydr-osugars in the chemical depolymerisation of heparin." Biochem., vol. 15(18), pp. 3932.

Silver, D.; Kapsch, D.N. and Tsoi, E. K. (1983) "Heparin induced thrombocytopenia, thrombosis and hemorrhage" Ann. Surg. vol 198(3) pp. 301-6.

Soeya, T. and Kono, R. (1957). "On some transition phenomena inaqueous solution of PVA." J. Phy. Soc. Japan, vol 12(8), pp.950-954.

Stivala, S.S.; Yuan, L.; Erlich, J. and Liberti, P.A. (1967)."Physiochemical studies of fractionated bovine heparin."Biochem. Biophys., vol. 112, pp. 32.

Sundberg, L. and Porath, J. (1974). "Preparation of adsorbents for biospecific affinity chromatography.

Attachment of group-containing ligands to insoluble polymers by means of bifunctional oxinanes." J. Chromatogr. vol. 90, pp. 87-98.

Suzuki, M.; Tamada, Y.; Iwata, H. and Ikada, Y. (1981). "Polymer surface modification to attain blood compatibility of hydrophobic polymer." In: K.L. Mittal (ed.), International Symposium on Physicochemical Aspects of Polymer Surfaces, Vol. 2, Plenum Press, New York.

Thunberg, L.; Backstrom, G.; Wasteson,A.; Robinson,H.C.; Ogren,S. and Lindhahl,U. (1982). "Enzymatic depo;ymerisation of heparin related polysaccharide" J. Biol. Chem. vol. 257(17) pp. 10278-82

Truce,W.E. and Norell, J.R.(1963) J. Amer. Chem. Soc. vol 85, pp. 3231.

Ushakov, S.N.; Trukhmanova, L.B.; Drozdova, E.V. and Markelova,T.M. (1961). Dokl. Akad. Nank. SSSR, vol. 141, pp. 1117.

Vroman,L. and Leonard, E.F.(1979) "The Behavior of Blood and its Components at Interfaces" ann of the NY Acad of Sci., publ. NY Acad. of Sci.

Wenning, M. and Hopker, W.W. (1981). "Scanning electron microscopic comparison between the thrombogenic properties of heparinised and non-heparinised vina cava catheters. A long term study." Virchows Arch [Pathol. Anat.], vol. 391(2), pp. 207-215.

White, J.G. and Burris, S.M. (1984). "Morphology of platelet internal contraction." Am. J. Pathol., vol. 115(3), pp. 412-7.

White, P.W.; Sadd, J.R. and Neusel, R.E. (1979). "Thrombotic complications of heparin therapy: including six cases of heparin-induced skin necrosis." Ann. Surg. vol. 190(5), pp. 595-608.

Wilchek, M.; Miron, T. and Kohn, J. (1984). "Affinity chromatography." In: Methods of Exzymology, Academic Press, vol. 104, pp. 3-55.

Wolf, R.M. and Suter, U.W. (1984). "Conformational characteristics of poly(vinyl alcohol)." Macromolecules, vol. 17, pp. 669-677.

Wong, R.F.; Windwer, S.R. and Feinman, R.D. (1983). "Interaction of thrombin and antithrombin. Reaction observed by

intrinsic fluorescence measurements." Biochemistry, vol. 22(17), pp. 3994-9.

Yahalom, J.; Eldor, A.; Fuks, Z. and Vlodavsky, I. (1984). "Degradation of sulphated proteoglycans in the subendothelial extracellular matrix by human platelet heparitinase." J. Clin. Invest. vol. 74(5), pp. 1942-9.

Appendix A

EVALUATION OF M_c FROM EQUILIBRIUM SWELLING EXPERIMENT (BRAY,1972)

This derivation is a modification of Flory's(1954) theory for networks crosslinked in bulk and swelled to equilibrium. In the development of this theory it is assume that the gel undergo affine deformation ie the amount of chain extention is proportional to the swelling of the gel. The modification takes into account the fact that the hydrogel were crosslinked in solution and was already in a somewhat expanded state when the crosslink junctions were introduced.

At equilibrium,

$$\mu_1 - \mu_1^o = 0 = \frac{\delta \Delta F}{\delta n_1} \qquad (1)$$

where

μ_1 = chemical potential of solvent in gel

μ_1^o = chemical potential of pure solvent

ΔF = free energy change

n_1 = moles of solvent

The change in free energy has two contribution: a mixing and elastic term.

$$\Delta F = \Delta F_{mix} + \Delta F_{el} \qquad (2)$$

The free energy of mixing of polymers in solvent is derived from Flory-Huggins lattice theory and is given by:

$$\Delta F_{mix} = kT\,[n_1 \ln v_1 + n_2 \ln v_2 + \chi_1 n_1 v_1] \qquad (3)$$

where

k = Boltzman constant

T = temperature in Kelvin

n_1 = moles of solvent

n_2 = moles of polymer chain

v_1 = volume fraction of solvent

v_2 = volume fraction of polymer

χ_1 = Flory-Huggins polymer - solvent interaction factor

For crosslink network $n_2 = 0$ and in the swollen state the above equation simplifies to :

$$\Delta F_{mix} = kT[n_1 \ln v_{1,s} + \chi_1 n_1 v_{2,s}] \quad (4)$$

Taking derivative wrt to n_1 and assuming simple additivity and remembering that the volume fractions are functions of n_1

$$\delta_{\frac{\Delta F_{mix}}{\delta n_1}} = kT[\ln(1-v_{2,s}) + v_{2,s} + \chi_1 v_{2,s}^2] \quad (5)$$

The free energy contribution of the elastic forces in the network is:

$$\Delta F_{el} = \Delta H_{el} - T\,\Delta S_{el} \quad (6)$$

In radiation crosslinking of a homopolymer ΔH_{el} may be assumed to be equal to 0. From statistical mechanics

$$S_{el} = \text{constant} - \frac{kv_e}{2}[\alpha x^2 + \alpha y^2 + \alpha z^2 - 3 - \ln(\alpha x \alpha y \alpha z)] \quad (7)$$

where S_{el} = elastic contribution to entropy

v_e = effective number of sub-chains in the network

$\alpha x, \alpha y, \alpha z$ = x, y, z contribution to the deformation from a relaxed state

The relaxed state in radiation crosslinked polymer in solution is taken to be the partially swollen state just

after irradiation where there are no stresses in the network. The elastic contribution as shown in equation 7 depends on the number of elastically effective subchains and the amount of deformation of these chains beyond its relaxed state.

where $\alpha^3 = \frac{V}{V_o}$

and V_o = volume at which crosslinks are introduced.

Assuming that the deformation is affine

$$\alpha = \alpha x = \alpha y = \alpha z$$

which yields

$$S = K - \frac{kv_e}{2}[3\alpha^2 - 3 - \ln\alpha^3]$$

$$= K - \frac{3kv_e}{2}[\alpha^2 - 1 - \ln\alpha]$$

$$\Delta S_{el} = S_{swelled} - S_{relaxed}$$

At relaxed state $\alpha = 1$

At swelled state $\alpha_s = V_s/V_r$

Substituting and rearranging gives

$$\Delta S_{el} = -\frac{3kv_e}{2}[\alpha_s^2 - 1 - \ln\alpha_s]$$

where α_s refers to the swollen state

$$\Delta F_{el} = \frac{3kTv_e}{2}[\alpha_s^2 - 1 - \ln\alpha_s]$$

Taking the derivative of ΔF_{el} with respect to n_1 and putting $\alpha_s = \alpha$:

$$\frac{\delta \Delta F_{el}}{\delta n_1} = \left(\frac{\delta \Delta F_{el}}{\delta \alpha} \right) \left(\frac{\delta \alpha}{\delta n_1} \right)$$

$$\alpha^3 = \frac{V_s}{V_r} = \frac{V_p + n_1 V_1}{V_r}$$

where V_p = volume of polyumer

n_1 = moles of solvent

V_1 = molar volume of solvent

Substituting this in and differentiating yields

$$3\alpha^2 \frac{\delta \alpha}{\delta n_1} = \frac{V_1}{V_r}$$

$$\frac{\delta \Delta F_{el}}{n_1} = \frac{3kTv_e}{2} \left[2\alpha - \frac{1}{\alpha} \right] \frac{V_1}{V_r} * \frac{1}{3\alpha^2}$$

$$= kTv_e \frac{V_1}{V_r} \left[\frac{1}{\alpha} - \frac{1}{2\alpha 3} \right]$$

$$\alpha^3 = \frac{V_s}{V_r} = \frac{v_{2,r}}{v_{2,s}}$$

$$\frac{\delta \Delta F_{el}}{\delta n_1} = kTv_e \frac{V_1}{V_r} \left[\left(\frac{v_{2,s}}{v_{2,r}} \right)^{1/3} - \frac{1}{2} \left(\frac{v_{2,s}}{v_{2,r}} \right) \right]$$

The effective number of sub-chains, V_e, must now be evaluated. An effective sub-chain is defined as a length of chain between two cross-link points. Consideration must be taken then of the number of free chain ends which are present.

Flory (1950) gives

$$v_e = v \left(1 - \frac{2M_c}{M_n} \right)$$

where v is the total number of sub-chains and the term in parentheses is Flory's correction for the free chain ends.

The total number of sub-chains is then the total weight of material originally present divided by the molecular weight between cross-links, M_c, or

$$v = \frac{N_0 M_0}{M_c}$$

where N_0 is the total number of monomer units and M_0 is the mean molecular weight of the monomer units.

It is also given by

$$v = \frac{V_p}{vM_c}$$

where V_p = volume of polymer (dry) present

v = specific volume of the polymer.

$$\frac{\delta \Delta F_{el}}{\delta n_1} = \frac{kTV_1}{v} \left(\frac{1}{M_c} - \frac{2}{M_n} \right) v_{2,r} \left[\left(\frac{v_{2,s}}{v_{2,r}} \right)^{1/3} - \frac{1}{2} \left(\frac{v_{2,s}}{v_{2,r}} \right) \right] \quad (8)$$

Combining eq (5) and (8)

$$\mu_1 - \mu_1^0 = kT[\ln(1 - v_{2,s}) + v_{2,s} + \chi_1 v_{2,s}^2] + \frac{kTV_1}{v} \left(\frac{1}{M_c} - \frac{2}{M_n} \right) v_{2,r} \left[\left(\frac{v_{2,s}}{v_{2,r}} \right)^{1/3} - \frac{1}{2} \left(\frac{v_{2,s}}{v_{2,r}} \right) \right]$$

$= 0$ at equilibrium.

This yields on rearrangement

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{\frac{v}{v_1}\left[\ln(1-v_{2,s}) + v_{2,s} + \chi_1 v_{2,s}^2\right]}{\left\{v_{2,r}\left[\left(\frac{v_{2,s}}{v_{2,r}}\right)^{1/3} - \frac{1}{2}\left(\frac{v_{2,s}}{v_{2,r}}\right)\right]\right\}}$$

If $v_{2,r}$ equals 1.0, i.e. if cross-links are introduced in the dry state, this relationship reduces to Flory's relation of

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{\frac{v}{v_1}\left[\ln(1-v_{2,s}) + v_{2,s} + \chi_1 v_{2,s}^2\right]}{\left(v_{2,s}^{1/3} - v_{2,s}/2\right)}$$

APPENDIX B

PROTEIN	MOL. WT.	RAD. OF GYRATION	REFERENCE
BSA	66,000	36.1 A	TANFORD,1961
OVALBUMIN	44,000	27.6 A	TANFORD,1961
CHYTRYPSINOGEN	23,200	22.5 A	TANFORD,1961
LYSOZYME	14,100	20.6 A	TANFORD, 1961
ANTITHROMBIN	52,000	-	*
THROMBIN	34,000	-	*

* - Determined experimentally by SDS-polyacrylamide gel electrophoresis (7.5%) and using Biorad stds

Appendix C

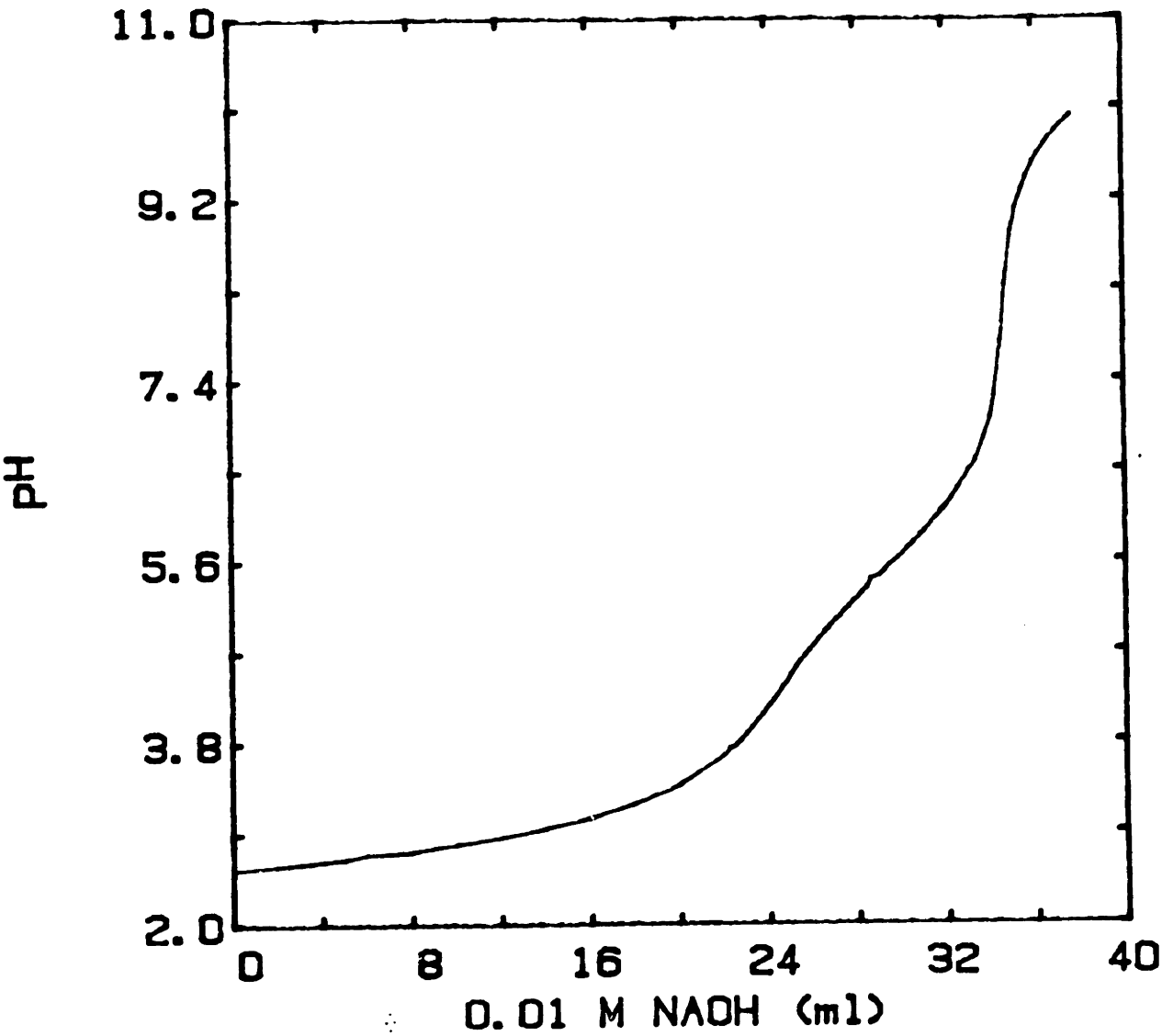


Figure 9-1 :Titration curve for Pure Heparin

APPENDIX D

Activation of PVA with Carbonyl Diimidazole

Figure 9-2 shows the swelling ratio of PVA in DMF after activation with carbonyl diimidazole. Only at the highest activation used did the swelling in DMF showed any appreciable increase. The swelling ratio of the activated PVA in water on the other hand decreases continuously with degree of activation (figure 9-3).

This is attributed to the change in the thermodynamics between the solvent and the activated PVA. χ which is the solvent polymer interaction factor increased to above 0.5 when the hydroxyls were replaced by the immidazole groups causing the decrease in the swelling of the gel.

The swelling ratio increased again when the immidazole group was replaced by the hydroxyl groups by reacting the activated gel with ethanolamine(figure 9-3). However the gels with the higher activation never really regain its initial swelling ratio probably because there are still a substantial amount of immidazole groups not replaced by ethanolamine in the interior matrix of the gel which would require a longer time for the ethanolamine to diffuse into it for complete inactivation.

Due to this phenomena CDI is not a suitable reagent to activate the hydroxyls of poly(vinyl alcohol). This phenomena

however was not observed with PEO and heparin has been successfully coupled to PEO using CDI.

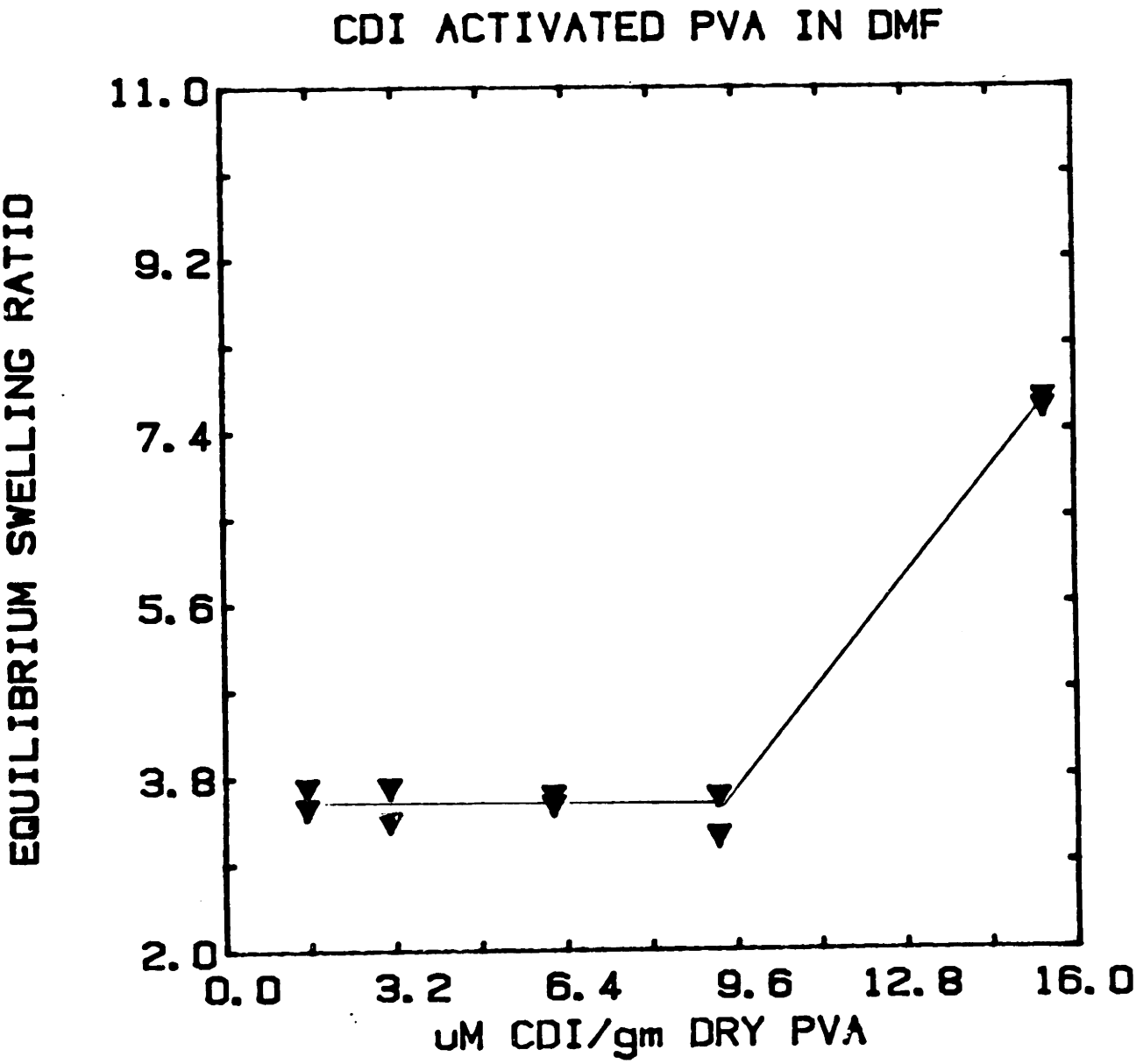


Figure 9-2: Swelling Ratio of CDI activated PVA in DMF

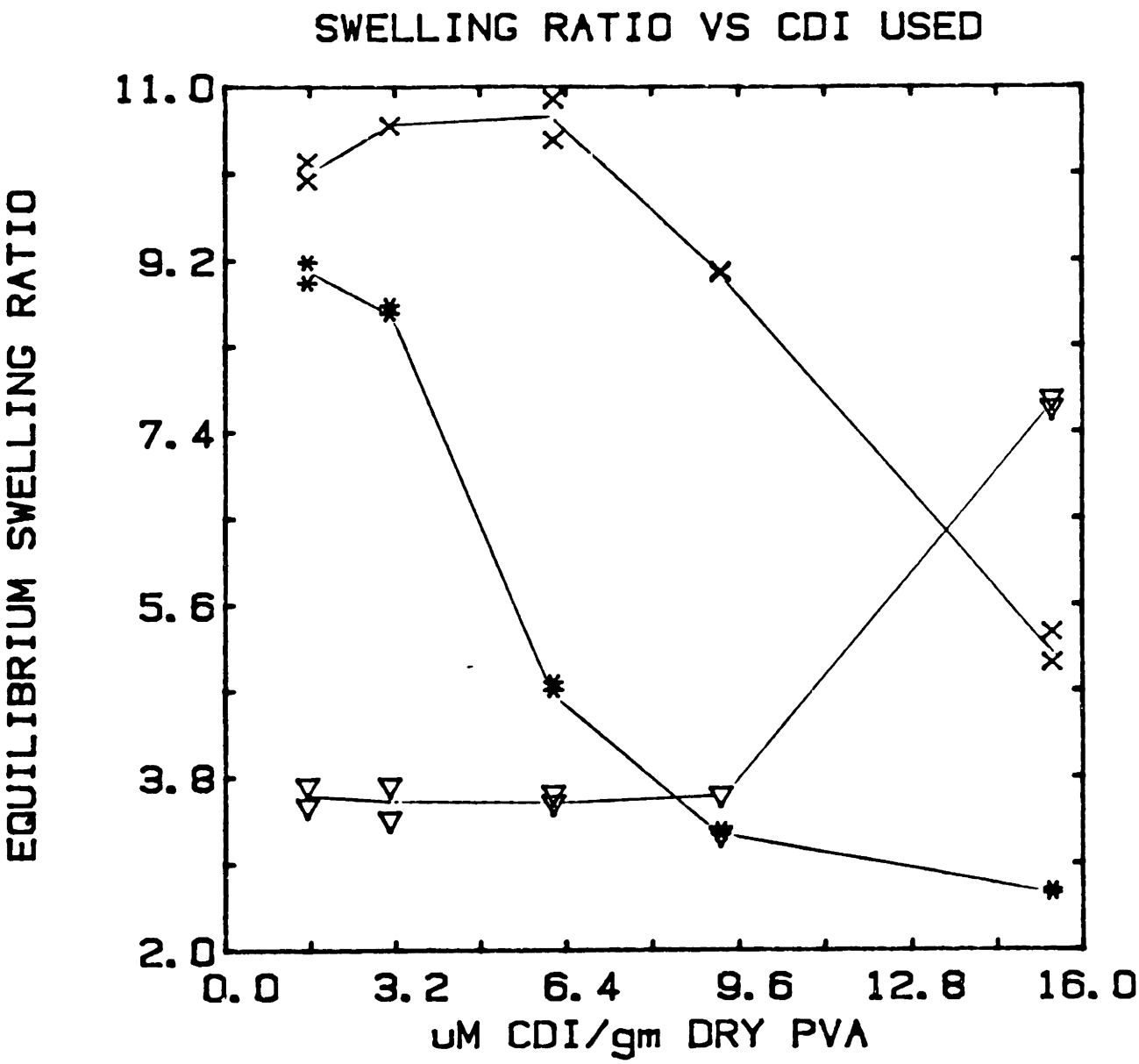


FIGURE9-3: EFFECT OF SOLVENT AND AMOUNT OF ACTIVATION BY CDI ON PVA

* ACTIVATED PVA IN WATER

▽ ACTIVATED PVA IN DMF

× PVA DEACTIVATED WITH ETHANOLAMINE IN WATER

APPENDIX F

Figure 9.4 below is a schematic diagram of a network showing the difference between the topological distance($\langle r^2 \rangle^{1/2}$) and the spatial distance (d_s) between junctions.

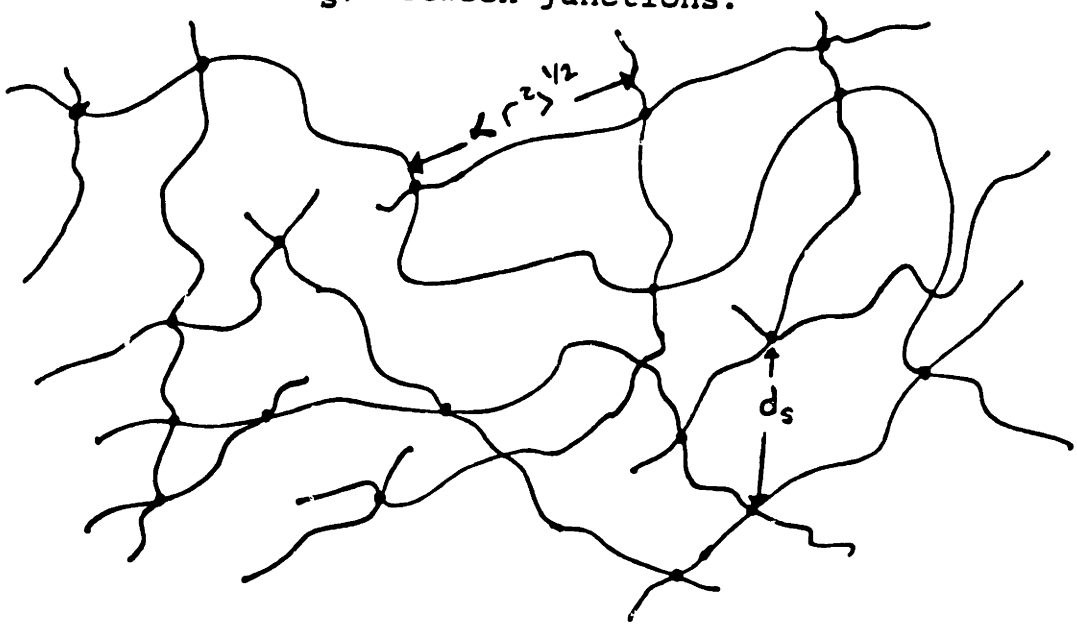


Figure 9.4 : Schematic Diagram of a ramdomly crosslinked network

From the equilibrium swelling measurements the M_c and v_{2s} values of the network can be calculated. Using these two values and the characteristic ratio of the polymer the two distances can be calculated as follows:

Topological distance ($\langle r^2 \rangle^{1/2}$)

In doing the following calculation it was assumed that the deformation is affine i.e. the expansion of the chain is proportional to the swelling of the network.

$\langle r^2 \rangle^{1/2} = \alpha \langle r^2_{} \rangle^{1/2}$

$\langle r^2 \rangle^{1/2} = (Cnl^2)^{1/2}$

$\alpha = (1/v_{2s})^{1/3}$

$n = X(M_c/M_o)$

where

α - is the expansion coefficient

v_{2s} - the polymer volume fraction in the swollen gel

C - characteristic ratio of the polymer

ℓ - average bond length (1.54 A for C-C bond)

n - the number of connecting the two junctions

X - the number of bonds in each repeat unit

M_o - the molecular weight of the repeating unit

M_c - the number average molecular weight between two topologically connected junction

$\langle r^2 \rangle^{1/2}$ - unperturbed end to end distance

Spatial Distance (d_s)

$n = (v_{2s} \times d \times N_A)/M_w$

$\mu = (M_n/M_c) + 1$

$d_s = (1/\mu n)^{1/3} \times 10^8$ angstroms

where

n - number of initial polymer molecule per ml gel

v_{2s} - volume fraction of polymer in the swollen gel

d - density of the polymer (gm/ml)

N_A - Avogardo's number

M_w - weight average molecular weight of the polymer

μ - number of junctions per polymer molecule

M_n - number average molecular weight of the polymer

M_c - number average molecular weight between two junctions

There is no text material missing here.
Pages have been incorrectly numbered.

181

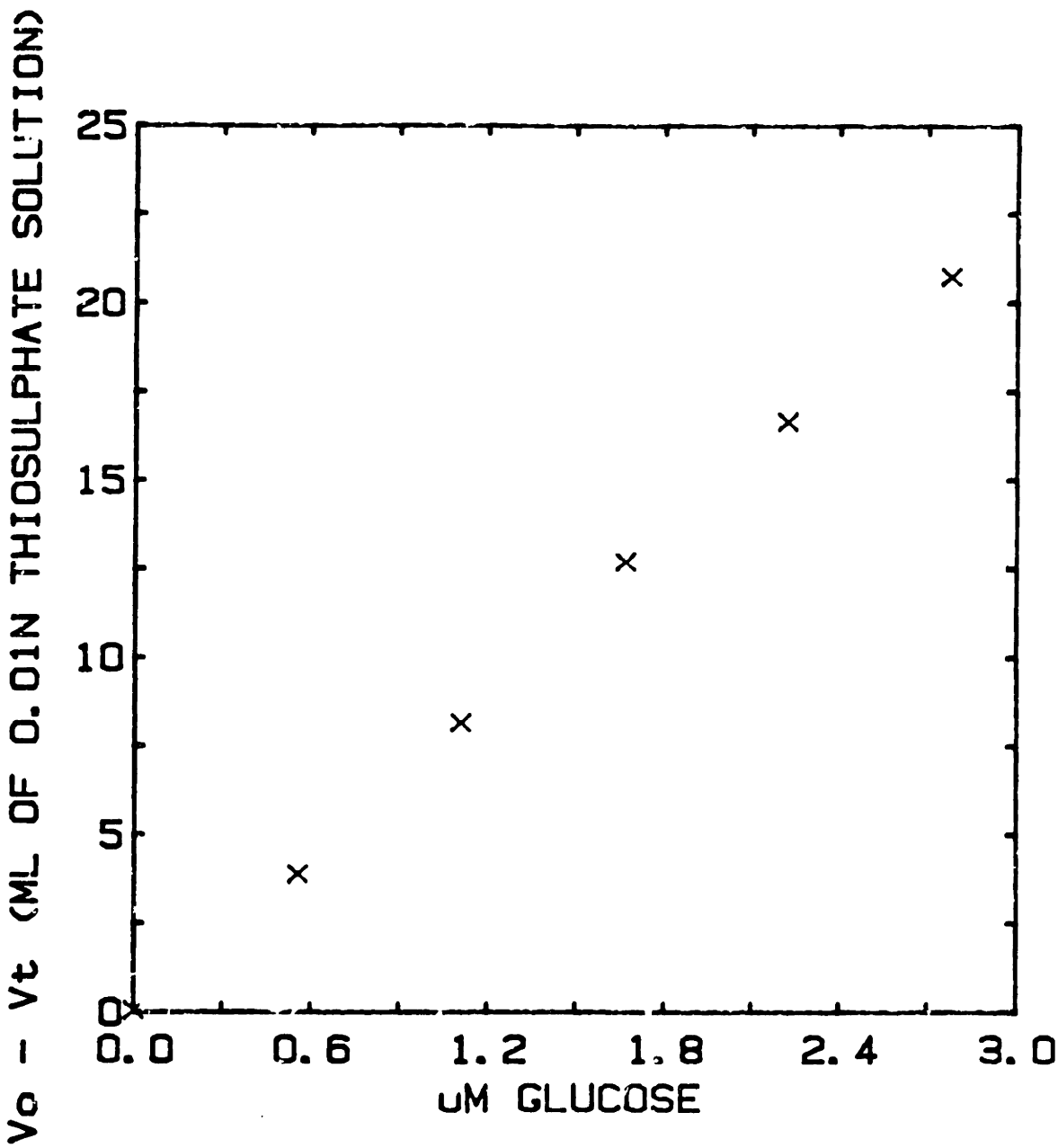


FIGURE 9-6: STANDARD CURVE FOR REDUCING SUGARS
Vo -VOLUME OF THIOSULPHATE TO NEUTRALISE BLANK
Vt - VOLUME OF THIOSULPHATE TO NEUTRALISE SAMPLE