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Subject Headings

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Azure A Assay	Heparin Structure	Heparin

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STUDIES ON IMMOBILIZED HEPARINASE AND THE HEPARINASE-HEPARIN REACTION

by

Gerald L. Fitzgerald

S.B. Chemical Engineering
Massachusetts Institute of Technology, June, 1982

S.B. Life Sciences
Massachusetts Institute of Technology, June, 1982

Submitted in Partial Fulfillment of
the Requirements for the Degree of

Master of Science in

Biochemical Engineering

at the
Massachusetts Institute of Technology

January 1983

Signature of Author.....Gerald L. Fitzgerald.....
Department of Nutrition and Food Science, January 13, 1983

Certified by.....Robert S. Langer.....
Robert S. Langer, Thesis Supervisor

Accepted by.....Steven R. Tannenbaum.....
Steven R. Tannenbaum, Chairman, Department Committee

1. ABSTRACT

STUDIES ON IMMOBILIZED HEPARINASE AND THE

HEPARINASE-HEPARIN REACTION

by

Gerald L. Fitzgerald

Submitted to the Department of Nutrition and Food Science on January 13, 1983, in partial fulfillment of the requirements for the degree of Master of Science in Biochemical Engineering.

Three related investigations of the heparinase-heparin reaction have been conducted. These investigations have been designed to reveal the sensitivity of the Azure A assay, the effect of immobilization of the enzyme on the reaction rate, and the distribution of the heparinase-cleavable sites in heparin.

Weight-basis standard curves for the Azure A assay were correlated for heparin fractions with different molecular weights. This correlation revealed that the Azure assay is sensitive to the weight of heparin in solution.

A reactor employing immobilized heparinase has been used by workers in this laboratory to continuously remove heparin from the bloodstream of dogs.[1] This reactor was modeled using theoretical correlations for the reaction rate and the mass transfer rate from the bulk solution to the surface of the beads used to immobilize the enzyme. This mathematical model of the reactor showed that the reactor was efficiently designed, with the exception of an unnecessary recirculation pump. The mathematical model was put into the form of a computer program to make the evaluation of proposed design changes simple.

Six different preparations of heparin having different known molecular weight distributions were degraded by soluble heparinase. The molecular weight distribution of the products formed by this reaction was found experimentally using gel permeation chromatography. This distribution of products was compared with distributions of products formed by computer simulations of heparin degradation that assumed the heparinase-cleavable sites in heparin to be randomly distributed in the heparin molecule. The two distributions were found to be identical to within a 99% confidence limit for all six preparations. Computer simulations of heparin degradation that assumed as little as 2% of a non-random distribution of heparinase-cleavable sites yielded final distributions of products significantly different from the distributions observed experimentally.

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1. Langer, R., R.J. Linhardt, S. Hoffberg, A.K. Larsen, C.L. Cooney, D. Tapper, and M. Klein, "An Enzymatic System for Removing Heparin in Extracorporeal Therapy," Science, 217, 261-263 (1982).

Thesis Supervisor: Professor Robert S. Langer
Title: Associate Professor of Biochemical Engineering

BIOGRAPHICAL NOTES

Born December 9, 1960 in West Reading, Pennsylvania, USA.

Education:
Massachusetts Institute of Technology Sept. 1978 to Jan. 1983
Received Bachelor of Science in Chemical Engineering and Bachelor of Science in Life Sciences simultaneously in June, 1982. Received 1982 Karl Taylor Compton Award for outstanding achievement and good citizenship, 1982 Elected Tau Beta Pi engineering honor society and served on the community service committee, 1981 John L. Asinari award for outstanding undergraduate research in the Life Sciences, 1981 named Seely Scholar for high achievement in chemical engineering, 1981 awarded NSF Summer Fellowship to do research at MIT on the design of the heparinase reactor.

I have been involved in a number of campus activities and held many leadership positions. Undergraduate Nominations Committee (4 yrs.) Chairman (2 yrs.), General Assembly (2 yrs.), Parliamentarian (1 year), MIT Educational Studies Program Teacher in Biology & Chemistry (3 yrs.), Secretary (1 year), Director (1 year), Alpha Phi Omega National Service Fraternity (3 yrs.), Administrative Vice President (1 year), Red Cross Water Safety and CPR Instructor (4 yrs.).

I have also contributed to the MIT Museum's activities. While working there I was responsible for cataloging and maintaining all the Audio-Visual equipment and materials. I also sorted through over 3000 MIT alumni obituaries to identify MIT alumni killed in the wars in Vietnam and Korea, edited a recorded collection of MIT songs for the album "Take Me Back to Tech", and compiled a history of the MIT Radiation Laboratory which included a complete personnel list for an epidemiological study on the effects of exposure to microwave radiation. I have been a member of the President's Advisory Committee to the Historical Collections and the Compton Gallery for 3 years.

Publication:
Robert J. Linhardt, Gerald L. Fitzgerald, Charles L. Cooney, and Robert Langer, "Mode of Action of Heparin Lyase on Heparin", Biochimica et Biophysica Acta, 702, 197-203 (1982).

Patent Application:
"Heparinase Derived Anticoagulants and Process" with Robert Langer, Arthur Grant, Robert Linhardt, and Charles Cooney

Undergraduate 24 unit Paper:
"Conditions, Kinetics, Mechanism, and Products of the Heparinase-Heparin Reaction"

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Thank you Prof. Robert S. Langer for your guidance and support over the years I have spent on this research, both as a graduate and undergraduate student. Without your positive criticism and enormous amount of support none of this thesis would have been possible.

Thank you Prof. Robert J. Linhardt for providing the hours and hours of conversation necessary to produce good ideas and the greater amount of time you gave to teaching me how to test those ideas.

Thanks to all my colleagues in the lab over the years: Michael Whelan, Steve Hoffberg, Laura Conway, Cynthia Zannetos, Cris Fitch, Parrish Galliher, and Arthur Grant as two heads and two pairs of hands always seem to get more work done in less time with less hassle. Special thanks to Arthur Grant, Ron Siegel, and Howard Bernstein for lending me their time and talents in the development of many ideas and for their comments on this manuscript.

To Tim Magee and Matt Lehman for late nights explaining mathematics.

To Maryanne Flaherty and Deirdre King for checking up on me when I didn't come home.

To Anne Armitage for keeping the Institute Screw at bay.

To Meg Morgan for making me eat meals, sleep regularly, have fun occasionally and all those other things one forgets about when writing a thesis.

This thesis is for my parents, Judith S. and Lawrence A. Fitzgerald, who worry for me, help me with the big decisions, and believe in me.

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2. INTRODUCTION

CLINICAL USE OF HEPARIN.

Heparin is an anticoagulant generally used during medical procedures that require the blood of a patient to come into contact with foreign surfaces. Most surfaces are not blood compatible, i.e., they cause blood to clot. The use of heparin allows the physician to use a whole range of extracorporeal devices that by necessity contain non-blood-compatible surfaces: from kidney dialysers to heart-lung machines to membrane oxygenators. All of these devices contain some surface (usually a membrane with specific permeabilities) which will cause the blood of the patient to clot. In order to prevent the blood from clotting inside these devices, heparin therapy is administered.

CURRENT HEPARIN THERAPY.

Current heparin therapy normally involves delivering a bolus injection of heparin to the patient at the start of the surgical procedure, followed by a slow intravenous drip. (See Figure 2-1) Maintaining the heparin level necessary to ensure blood compatibility is fairly easy, since the body degrades heparin's anticoagulant activity very slowly.

PROBLEMS WITH HEPARIN THERAPY.

The major problem with current heparin therapy arises after the surgical procedure is over. Because heparin is cleared so slowly, the patient's blood does not return to its normal coagulation state for 6 to 8 hours. During this time the patient is at risk of hemorrage and complications resulting from the oozing of blood. Usually the patient is allowed to clear the heparin on his own; however, for certain high-risk patients protamine sulphate is injected into the patient following the procedure. While protamine is a heparin antagonist and quite effective in reducing the activity of heparin, it is also toxic. It is estimated that between 8 and 33% of all surgical procedures

CURRENT HEPARIN THERAPY

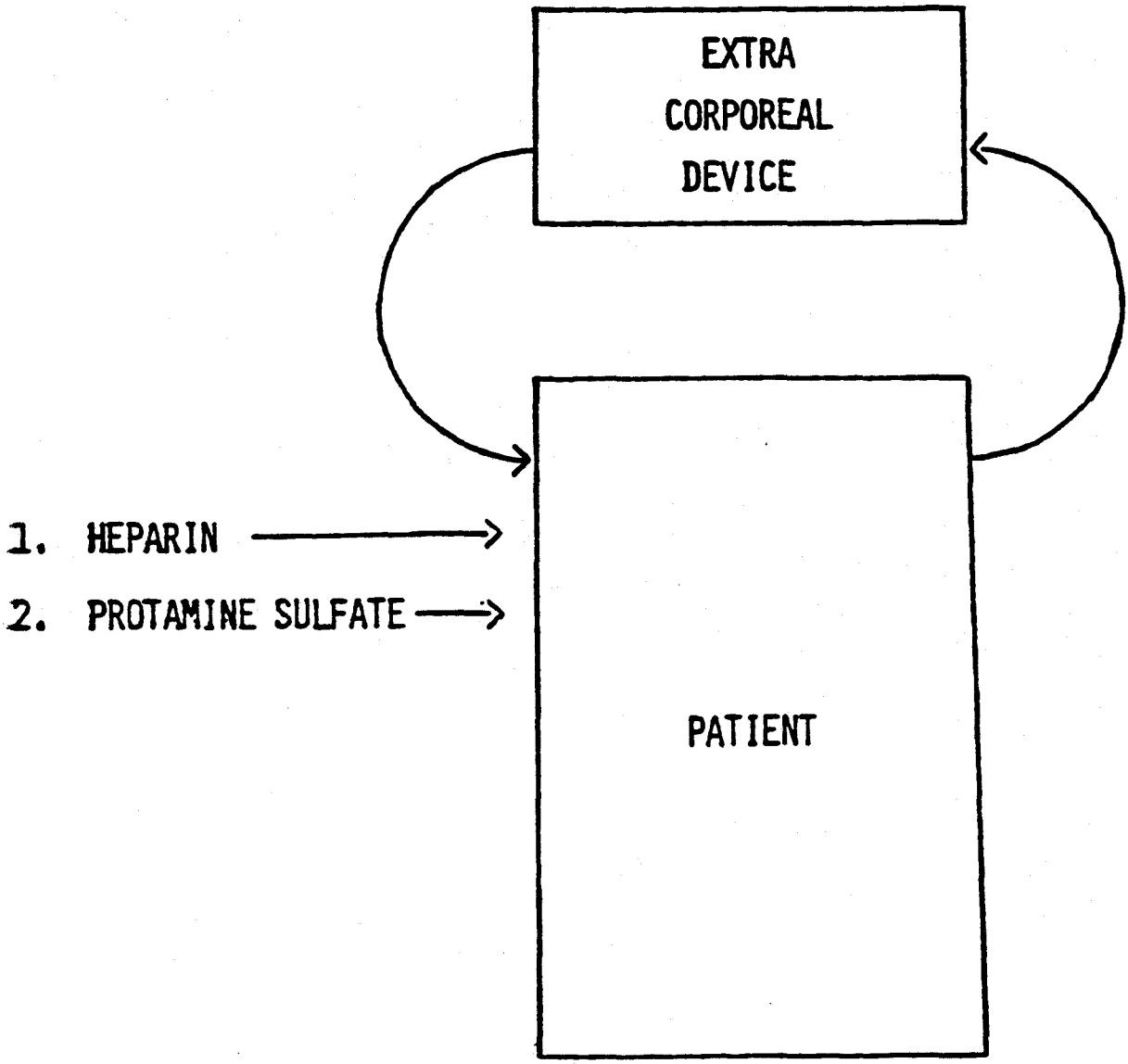

FIGURE 2-1

involving heparin experience some heparin complications.[2] Especially at risk are diabetics, for whom retinopathy is a distinct danger.

PROPOSED HEPARIN THERAPY.

These problems have led researchers in our laboratory to consider a novel approach to heparin therapy.[3] This approach uses an immobilized microbial heparinase to remove heparin from the bloodstream. The proposed use of this device is to continuously remove heparin from the bloodstream after it leaves the device and before it enters the patient. This configuration (shown in Figure 2-2) allows the heparin to be present in the extracorporeal device, where it has therapeutic value, and not in the patient, where it is potentially harmful. Since the patient is not exposed to heparin, the level of heparin in extracorporeal circulation can be increased, reducing the hazards of clotting without increasing any hazard to the patient. In fact, for certain procedures, the hazards to the patient can be reduced tremendously. A heparinase reactor of the type proposed has been successfully used to remove heparin from the bloodstream of a dog.[4] This reactor operated as a continuous stirred tank reactor using immobilized heparinase.

SOURCE OF HEPARINASE.

Heparinase is an inducible, non-extracellular enzyme produced by Flavobacterium heparinum. This bacterium was originally selected by Korn and

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2. Gervin, A.S., "Complications of Heparin Therapy", Surg. Gynecol. Obstet., 140, 789-796 (1975).

3. Langer, R., R.J. Linhardt, M. Klein, M.M. Flanagan, P.M. Galliher, C.L. Cooney, "A System for Heparin Removal," in Biomaterials: Interfacial Phenomena and Applications, S. Cooper, A. Hoffman, N. Peppas, B. Rattner, eds., Washington, D.C., American Chemical Society, 493-509 (1982).

4. Langer, R., R.J. Linhardt, S. Hoffberg, A.K. Larsen, C.L. Cooney, D. Tapper, and M. Klein, "An Enzymatic System for Removing Heparin in Extracorporeal Therapy," Science, 217, 261-263 (1982).

PROPOSED HEPARIN THERAPY

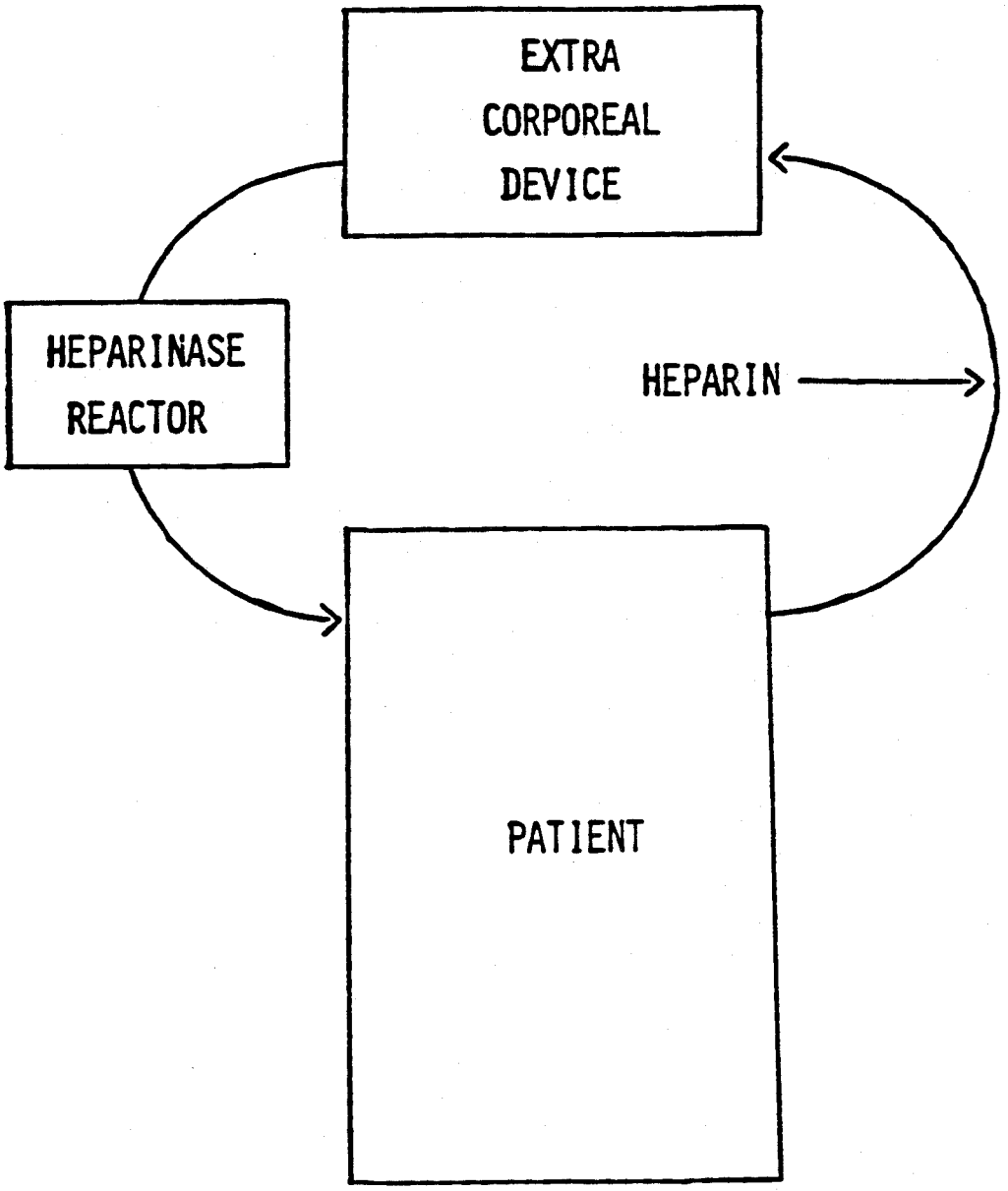

FIGURE 2-2

Payza in 1956 from soil bacteria for its ability to utilize heparin as a sole source of carbon, nitrogen, and sulphur.[5] The growth requirements and heparinase production of the bacteria were reported by previous workers in this laboratory.[6] The first studies done on the particular enzymes responsible for heparin degradation were done by Linker and Hovingh.[7] Later studies showed that heparinase was the first of 5 enzymes used by F. heparinum to degrade heparin. The other enzymes produced included glycuronidases, sulfoesterases, sulfamidases, and heparatinase (heparin monosulfate lyase).[8] Heparinase is purified using a variety of methods, including hydroxylapatite, isoelectric focusing, chromatofocusing, and cellulose phosphate. The enzyme has recently been purified by my colleague, C. Zannetos.[9]

PROPERTIES OF HEPARINASE.

The properties of heparinase are ideal for its operation in the bloodstream. Heparinase [heparin lyase E.C. 4.2.2.7] is an alpha 1,4 eliminase. It is specific for alpha linkages between N-sulphated D-glucosamine (the 6-, or O-sulphate may not be required) and sulphated

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5. Korn, E.D. and A.N. Payza, "Bacterial Degradation of Heparin", Nature (London), 177, 88-89 (1956).

6. Galliher, P.M., C.L. Cooney, R. Langer, and R.J. Linhardt, "Heparinase Production by Flavobacterium heparinum," Appl. Environ. Microbiol., 41, 360-365 (1981).

7. Linker, A., and P. Hovingh, "Isolation and Characterization of Oligosaccharides Obtained from Heparin by the Action of Heparinase," Biochemistry, 11, 563-568 (1972).

8. Dietrich, C.P., M.E. Silva, and Y.M. Michelacci, "Sequential Degradation of Heparin in Flavobacterium heparinum," J. Biol. Chem., 248, 6408-6415 (1973).

9. Zannetos , C., "The Purification of Heparinase from Flavobacterium Heparinum," S.M. Thesis, MIT, March, 1983.

L-iduronic acid. Heparinase will not cleave a particular alpha linkage if the amine group is acetylated or if the sulphate group on the iduronic acid is missing.[10] The enzyme has a broad activity vs. pH maximum centered around pH 6. The maximum stability of the enzyme is achieved at pH 7. The activity of the enzyme is a maximum at an ionic strength of solution of 0.15 M.[11] The enzyme is unaffected by the presence of most cations, with the notable exceptions of Ca^{++}, Cd^{++}, and Hg^{++}.[12] The enzyme is not inhibited by other polysaccharides, however, highly sulphated polymers such as polystyrene sulfonic acid, polyvinylsulfate and polyanethole sulfate all inhibit the activity of the enzyme. All three of these substances are artificial anticoagulants.[13] Heparinase has not shown product inhibition at initial product concentrations as high as 50 mg/ml. The enzyme has been shown to have a random endolytic mode of action.[14]

IMMOBILIZATION OF HEPARINASE.

Before the enzyme can be used in the bloodstream, it must first be immobilized. Immobilization reduces the risk of antigenic complications arising from contact between microbial proteins and human blood.[15]

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10. Hoffberg, S.N., "A System for Enzymatic Blood Deheparinization," S.M. Thesis, MIT, Sept., 1981.

11. See Appendix D

12. See Appendix E

13. Flanagan, M.M., "Purification and Characterization of Heparinase," S.M. Thesis, MIT, March, 1981.

14. Linhardt, R.J., G.L. Fitzgerald, C.L. Cooney, and R. Langer, "Mode of Action of Heparin Lyase on Heparin," Biochim. Biophys. Acta, 702, 197-203 (1982).

15. Langer, R., R.J. Linhardt, C.L. Cooney, D. Tapper, and M.D. Klein, "Immobilized Heparinase: Production, Purification, and Application in Extracorporeal Therapy," in Enzyme Engineering, I. Chibata, S. Fukui, and L.B. Wingard, eds., New York, Plenum Press, p. 433-441 (1982).

Immobilization of an enzyme often also increases the stability of the enzyme, and allows one to have better control over the reaction by limiting the time of contact between substrate and enzyme. An immobilized enzyme system for use in the bloodstream must have high enzymatic activity, it must be blood compatible, and it must be stable under the conditions in the bloodstream. High activity is necessary to keep the reactor small, so that only a small volume of blood is necessary to fill it. Blood compatibility is needed so that the surgical procedure is not further complicated by the use of the device. The enzyme activity must be stable over the duration of the surgical procedure, and no part of the enzyme or support should leach into the bloodstream, possibly causing toxic side effects.

Heparinase has been successfully immobilized to Sepharose 4B® using a cyanogen bromide linkage.[16] After immobilization, over 90% of the specific activity of the heparinase is retained. Sepharose is marginally blood compatible.[17] The half life of the enzyme at 30 ℃ is 1000 hours. Leaching of the enzyme from the support is a small problem, as less than 2% of the protein is detectable in solution after 1 month.[18] Sepharose 4B has a high capacity for enzymes, 2 umol per mg gel, allowing for a high concentration (0.1 mg heparinase per ml gel) of enzyme in the support.[19]

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16. Linhardt, R.J., G.L. Fitzgerald, C.L. Cooney, and R. Langer, "Mode of Action of Heparin Lyase on Heparin," Biochim. Biophys. Acta, 702, 197-203 (1982).

17. Hoffberg, S.N., "A System for Enzymatic Blood Deheparinization," S.M. Thesis, MIT, Sept., 1981.

18. ibid.

ASSAYS FOR HEPARIN AND HEPARIN ANTICOAGULANT ACTIVITY.

Three types of assay are used for heparin and heparin activity. These three types measure the amount of heparin in solution, the amount of heparin degradation products in solution, and the anticoagulant activity of the solution. The usual assay for direct measurement of heparin in solution is the Azure A assay. The UV 232 nm assay measures the amount of a chromophore present in heparin degradation products. Anticoagulant activity methods measure heparin concentration via the magnitude of the effect of the sample on either clotting time or a particular reaction in the clotting cascade.

THE AZURE A ASSAY.

The Azure A assay is widely used to measure the concentration of heparin in solution. Colorimetric reactions with various dyes have been used in non-protein-containing solutions for determination of non-degraded heparin.[20] The Azure A assay has proven to be precise enough to have been proposed as a method for standardization of heparin units.[21] Jacques has studied the metachromatic reaction of heparin with both Azure A and toluidine blue[22] and has proposed that Azure A dye molecules dimerize in the presence of heparin

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19. Pharmacia Fine Chemicals Company, Affinity Chromatography: Principles and Methods. Pharmacia Fine Chemicals, Uppsala, Sweden.

20. Anderson, W., J.E. Harthill, and R.H. Price-Jones, "A Comparison of Three Chemical Methods of Assay of Heparin," J. Pharm. Pharmacol., 31(Suppl.), 93 P (1979).

21. Copley, A.L., and D.V. Whitney III, "The Standardization and Assay of Heparin by the toluidine blue and Azure A reactions," J. Lab. Clin. Med., 28, 762-770 (1943).

22. Jacques, L.B.,"Determination of Heparin and sulfated mucopolysaccharides," Methods Biochem. Anal., 24, 203-321 (1977).

resulting in a decrease in the pi delocalization. This is observed as a shift in the absorbance maxima of the dye molecules from λ_{max} = 620nm to λ_{max} = 520nm.[23] Since heparinase cleaves the alpha linkage of heparin, its action causes chain shortening resulting in less metachromasia. The presence of heparin or heparin-like polysaccharides of hexasaccharide length or longer[24] can be measured reproducibly at levels of 1 to 10 mg/ml in crude and 1 to 10 ug/ml in purified preparations. The Azure A assay has been used effectively in crude bacterial sonicates to follow heparinase production in Flavobacterium heparinum.[25] Recently, the Azure assay has also been adapted for clinical use in human plasma.[26]

THE UV 232 NM ASSAY.

The molar amount of products was assayed by absorbance at UV 232nm. Because heparinase is an alpha 1,4-eliminase, it cleaves heparin leaving an α,β unsaturated endgroup. This chromophore has a maximum absorbance at approximately 232nm and a molar extinction coefficient of about 5.5×10^3 Optical Density units per mole per liter-cm.[27] The concentration of heparin

23. Jacques, L.B., "Heparin: An Old Drug with a New Paradigm," Science, 206, 528-533 (1979).

24. Dietrich, C.D., "Novel Heparin Degradation Products," Biochem. J., 108, 647-654 (1968).

25. Galliher, P.M., C.L. Cooney, R. Langer, and R.J. Linhardt, "Heparinase Production in Flavobacterium heparinum," Appl. Environ. Microbiol., 41, 360-365 (1981).

26. Klein, M.D., R.A. Drongowski, R.J. Linhardt, and R.S. Langer, "A Colorimetric Assay for Chemical Heparin in Plasma," Anal. Biochem., 124, 59-64 (1982).

27. Linker, A. and P. Hovingh, "Isolation and Characterization of Oligosaccharides obtained from Heparin by the Action of Heparinase," Biochemistry, 11, 563-568 (1972).

products is followed by diluting the reaction mixture to about 0.1 mg/ml of product in distilled water and measuring the optical absorbance. This assay method can be used only for purified enzyme preparations. In crude enzyme preparations the high concentration of protein lowers the assay's sensitivity and contaminating enzymes, especially glycuronidases, catalyse the loss of the chromophore. This assay is recommended for its simplicity, enabling the experimenter to run many reactions simultaneously.

COAGULATION ASSAYS.

Four assays are commonly used to measure the anticoagulant activity of a heparin sample in our laboratory. Two are clotting methods: the activated partial thromboplastin time and the Factor X_a clotting time. The other two are chromogenic assays and use synthetic substrates to measure heparin-induced inactivation of proteases in the clotting cascade.

APTT.

The activated partial thromboplastin clotting time (APTT) is a clinical test for the effect of various factors on the intrinsic clotting system. the APTT is sensitive to clinically used concentrations of heparin (.05 to .5 units/ml). The APTT involves a plasma recalcification clotting time, which is modified to control for the activation of the surface contact factor (Hageman III factor). This activation is accomplished by the addition of phospholipids (usually acetone brain extract) which replace platelet phospholipids (thromboplastin) in the activation of the clotting mechanism. These steps ensure maximum activation of Factor X if the coagulation cascade is intact.[28]
29 30 31 32 33 34 35 36 37

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28. Harker, L.A., Hemostasis Manual, 2nd ed., Philadelphia, F.A. Davis Co. (1976).

FACTOR X_a CLOTTING TIME.

The Factor X_a assay is based on the inhibitory action of the heparin-antithrombin III complex on activated factor X_a.[38 39] Heparin acts to greatly accelerate the inhibitory action of antithrombin III (AT-III) on specific blood serine proteases. In this assay, a measured amount of

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29. Williams, Beutler, Erslev, and Rundles (eds.), Hematology, 2nd ed. New York, McGraw-Hill, p. 1285-1293 and 1641-1649 (1977).

30. Briselli, M.F., and L. Ellman, "Kaolin-correctable prolongation of the activated partial thromboplastin time," Am. J. Clin. Pathol., 72, 677-680 (1980).

31. Orthodiagnostics, Inc., "Activated Throbofax Reagent (Partial Thromboplastin with Activator) for the Activated Partial Thromboplastin Time Test." Raritan, N.J., Orthodiagnostics, Inc. (1980)

32. Barez, E.I., D.A. Tiplett, and J. Koepke, "Laboratory monitoring of heparin therapy--the effect of different salts of heparin on the activated partial thromboplastin time," Am. J. Clin. Pathol., 72, 569-574 (1980).

33. Ts'ao, C.H., T.S. Galluzzo, M.T. Roselo, and K. Peterson, "Whole-blood clotting time, activated partial thromboplastin time, and whole-blood recalcification time as heparin monitoring tests," Am. J. Clin. Pathol., 71, 17-21 (1979).

34. Bain, B., T. Forster, and B. Sleigh, "Heparin and the activated partial thromboplastin time--a difference between the in vitro and in vivo effects and implications for the therapeutic range," Am. J. Clin. Pathol., 74, 668-673 (1980).

35. Babka, R., C. Colby, A. El-Etr, and R. Pifarre, "Monitoring of intraoperative heparinization and blood loss following cardiopulmonary bypass surgery," J. Thorac. Cardiovasc. Surg., p.780-782 (1977).

36. Shapiro, G.A., S.W. Huntzinger, and J.E. Wilson, "Variation among commercial activated partial thromboplastin time reagents in response to heparin," Am. J. Clin. Pathol., 67, 477-480 (1977).

37. Teien, A., and M. Lie, "Heparin assay in plasma: a comparison of five clotting methods," Thromb. Res., 67, 789-795 (1975).

38. Hillman, R.S., and C.A. Finch, Red Cell Manual, 4th ed., Philadelphia, F.A. Davis Co. (1976).

39. Sigma Chemical Company, "A Clotting Procedure for the Quantitative Determination of Heparin in Plasma," Technical Bulletin #870 (1977).

activated factor X_a is added to a citrated plasma sample containing an unknown amount of heparin. The heparin is allowed to interact with the antithrombin III (Factor X_a inhibitor) present in the plasma sample for a set amount of time, and then the clotting time is measured by recalcifying the sample and initiating clotting with plasma CEF (cephalin from rabbit brain in anticoagulant-free bovine plasma), a platelet phospholipid (thromboplastin) substitute. The clotting time represents the amount of factor X_a remaining in the sample, and when performed as originally described by Yin et. al., the elongation in clotting time is linear with heparin activity up to .25 units per ml of sample.[40 41] At heparin activities over .25 units/ml the added factor X_a is consumed, leaving some heparin-antithrombin III to act on other blood factors. The factor X_a assay measures heparin activity by its coordinated action with AT III on factor X_a. The activities obtained do not correlate well with the net quantity of heparin measured by the Azure A assay after degradation by heparinase.[42]

THROMBIN INHIBITION.

Thrombin can be measured in plasma using a chromogenic substrate. The synthetic tripeptide used in this assay is H-D-phe-pro-arg-5-amidoisophathalic acid dimethyl ester. This assay displays sensitivity and accuracy better than the clotting methods because the high dilution eliminates the clotting interference usually found in thrombin assays (thrombin normally acts on

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40. Hillman, R.S., and C.A. Finch, Red Cell Manual, 4th ed., Philadelphia, F.A. Davis Co. (1976).

41. Sigma Chemical Company, "A Clotting Procedure for the Quantitative Determination of Heparin in Plasma," Technical Bulletin #870 (1977).

42. Hoffberg, S.N., "A System for Enzymatic Blood Deheparinization," S.M. Thesis, MIT, Sept., 1981.

fibrinogen).[43] It has been proposed that the thrombin-antithrombin III reaction is more affected by heparin than the Factor X_a-ATIII reaction.[44] This makes the chromogenic thrombin assay very attractive for measurement of heparin activity. Also, the products of enzymatic degradation of heparin act with Antithrombin III to inhibit Factor X_a, while the ATIII-heparin product complex does not inhibit thrombin.[45]

FACTOR X_a INHIBITION.

The chromogenic Factor X_a assay, using the synthetic substrate benzoyl-ile-glu-gly-arg-p-nitroanilide is much more accurate than the clotting assay (2% standard deviation versus 9%) on normal plasmas and 8% versus 14% in pathological samples with a similar range of sensitivities (0.02 to 0.25 versus 0.01 to .0.25 units/ml for the clotting assay).[46] [47]

VARIATION OF HEPARIN ACTIVITY WITH MOLECULAR WEIGHT.

When polydisperse heparin preparations have been fractionated by molecular weight, it has been found that the higher molecular weight fractions

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43. Mitchell, G.A., R.J. Cargiulo, R.M. Huseby, D.E. Lawson, S.P. Pochron, and J.A. Sehvanes, "Assay for plasma heparin using a synthetic peptide substrate for thrombin: Introduction of the fluorophore aminosulphthalic acid, dimethyl ester," Thromb. Res., 13, 47-52 (1978).

44. ibid.

45. Hoffberg, S.N., "A System for Enzymatic Blood Deheparinization," S.M. Thesis, MIT, Sept., 1981.

46. Hasegawa, H., Y. Oguma, H. Takei, T. Sega, M. Yamauchi, T. Murakoshi, H. Nagata, and M. Murao, "Assay of heparin in plasma using a chromogenic substrate and its clinical applications," Jpn. Heart J., p. 367-380 (May 1980).

47. Teien, A.N., M. Lie, and U. Abildgaard, "Assay of heparin using a chromogenic substrate for activated factor X," Thromb. Res., 8, 413-416 (1976).

contain a disproportionate amount of the anticoagulant activity of the heparin sample.[48 49 50 51 52 53 54 55] The results of Hopwood et al. imply that antithrombin binds to a specific site on the heparin molecule and that the anticoagulant activity of heparin should be related to the probability of finding this site in the molecules of the preparation.[56] The biosynthesis of heparin is initiated by polymerization of alternating N-acetyl-D-glucosamine and D-glucuronic acid residues. Various modifications to the disaccharides

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48. Laurent, T.C., "Studies on Fractionated Heparin," Arch. Biochem. Biophys., 92, 224-231 (1961).

49. Lasker, S.E., and S.S. Stivala, "Physicochemical Studies of Fractionated Bovine Heparin: Some Dilute Solution Properties," Arch. Biochem. Biophys., 115, 360-372 (1966).

50. Liberti, P.A., and S.S. Stivala, "Physicochemical Studies of Fractionated Bovine Heparin: Viscosity as a Function of Ionic Strength," Arch. Biochem. Biophys., 119, 510-518 (1967).

51. Stivala, S.S. and P.A. Liberti, "Physicochemical Studies of Fractionated Bovine Heparin: Cu(II) Binding in Relation to pH, Molecular Weight, and Biological Activity," Arch. Biochem. Biophys., 122, 40-52 (1967).

52. Cifonelli, J.A., "The Relationship of Molecular Weight, and Sulfate Content and Distribution to Anticoagulant Activity of Heparin Preparations," Carbohyd. Res., 37, 145-154 (1974).

53. McDuffie, N.M., C.P. Dietrich, and H.B. Nader, "Electrofocusing of Heparin: Fractionation of Heparin into 21 Components Distinguishable from Other Acidic Mucopolysaccharides," Biopolymers, 14, 1473-1486 (1975).

54. Andersson, L.O., T.W. Barrowcliffe, E. Holmer, E.A. Johnson, and G.E.C. Sims, "Anticoagulant properties of heparin fractionated by affinity chromatography on matrix-bound antithrombin III and by gel filtration," Thromb. Res., 9, 575-583 (1976).

55. Riesenfeld, J., M. Hook, I. Bjork, U. Lindahl, and B. Ajaxon, "Structural requirements for interaction of heparin with antithrombin III," Fed. Proc. Fed. Am. Soc. Exp. Biol., 36, 39-43 (1977)

56. Hopwood, J., M. Hook, A. Linker, and U. Lindahl, "Anticoagulant Activity of Heparin: Isolation of Antithrombin-Binding Sites," FEBS Lett., 69, 51-54 (1976).

are introduced at the polymer level.[57] The molecular weight dependence of heparin activity toward antithrombin III might be explained by a probabilistic model that assumes a random distribution of different disaccharides.[58] Such a random distribution of different disaccharides would specify that the heparinase-cleavable alpha linkages of heparin would be distributed in a random, independent manner.

GOALS OF THE CURRENT INVESTIGATION.

Three related investigations of the heparinase-heparin reaction have been conducted. These investigations have been designed to reveal the sensitivity of the Azure A assay, the effect of immobilization of the enzyme on the reaction rate, and the distribution of the heparinase-cleavable sites in heparin.

The sensitivity of the Azure A assay--whether to moles or weight of heparin in solution--has not been previously determined. While the Azure A assay does correlate very well with anticoagulant assays,[59] the correlation may depend on the molecular weight of the heparin being used. While standard curves for the Azure A assay are normally done in terms of weight concentration of heparin for convenience, no correlation has been made between

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57. Lindahl, U., M. Hook, G. Backstrom, I. Jacobsson, J. Riesenfeld, A. Malmstrom, L. Roden, and D.S. Feingold, "Structure and Biosynthesis of heparin-like polysaccharides," Fed. Proc. Fed. Am. Soc. Exp. Biol., 36, 19-23.

58. Laurent, T.C., A. Tengblad, L. Thunberg, M. Hook, and U. Lindahl, "The Molecular-Weight-Dependence of the Anti-Coagulant Activity of Heparin," Biochem. J., 175, 691-701 (1978).

59. Klein, M.D., R.A. Drongowski, R.J. Linhardt, and R.S. Langer, "A Colorimetric Assay for Chemical Heparin in Plasma," Anal. Biochem., 124, 59-64 (1982).

the various standard curves obtained for different molecular weight heparins. The correlation is important to the usefulness of the assay across different heparin preparations. If the Azure A assay were sensitive to moles of heparin, for instance, the Azure A assay would underestimate the anticoagulant activity of large molecular weight heparins.

An enzymatic reactor employing immobilized heparinase has been used *in vivo* to continuously remove heparin from the bloodstream of dogs.[60] In order to optimize the design of this reactor for efficient operation, it is necessary to understand the physical laws governing the reaction between immobilized heparinase and heparin in solution. This includes determining whether the rate-limiting step in the reaction is the reaction itself or some mass transfer step, either from the bulk solution to the surface of the bead support, or within the bead itself. Once an accurate model for the immobilized enzyme system has been constructed, it can be used to recommend optimal bead size and enzyme loading, type of bead, and rate of stirring.

The distribution of heparinase-cleavable sites in the heparin polymer contains many theoretical implications about the reaction and the structure of heparin. First of all, the distribution of heparinase-cleavable sites would explain why a particular final distribution of products is obtained when heparin is degraded by heparinase. Assuming that the heparinase-cleavable sites are somehow chemically different from heparinase-uncleavable sites (which is implied by both enzyme specificity and the consistent product distributions obtained with different preparations of heparin and heparinase,

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60. Langer, R., R.J. Linhardt, S. Hoffberg, A.K. Larsen, C.L. Cooney, D. Tapper, and M. Klein, "An Enzymatic System for Removing Heparin in Extracorporeal Therapy," Science, 217, 261 (1982).

even after immobilization of the enzyme)[61] the distribution of heparinase-cleavable sites would also imply some aspects of heparin structure. Including the affinity between heparinase and different sized chains of heparin polymers, the molecular weight distribution of heparin over time can be predicted. Being able to predict the molecular weight distribution over time is a first step toward being able to predict the anticoagulant activity over time. The complete model for anticoagulant activity would have to include both the chemical structures for heparin's heparinase-cleavable and anticoagulant sites.

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61. Linhardt, R.J., A. Grant, C.L. Cooney, and R. Langer, "Differential Anticoagulant Activity of Heparin Fragments Prepared Using Microbial Heparinase", J. Biol. Chem., 257, 7310-7313 (1982).

3. THEORY

THE AZURE A ASSAY.

What has not been explored thus far about the Azure A assay is whether the assay is sensitive to molar concentration or weight concentration of heparin. This may be due to the scarcity of size-fractionated heparin. By carefully assaying measured weights of heparins of different molecular weights over the range from 8,000 to 20,000 daltons, the sensitivity of the assay is easily determined. Over the range of 1 to 10 ug/ml of heparin, the metachromasia of Azure A solutions is linear at UV 620nm for heparins of this moderate size.[62] If the assay is sensitive to the weight of heparin in solution, then standard curves made on a weight basis with different molecular weights of heparin should be identical. If the assay is sensitive to the number of moles of heparin in solution, then the slope of these standard curves should vary with the molecular weight of the heparin used.

IMMOBILIZED ENZYME KINETICS.

Immobilized enzymes are intriguing scientifically for at least three reasons:

1. Their study can lead to new insight on the in vivo operation of enzymes, since many enzymes are membrane bound inside the cell.

2. The reaction catalysed is part of an industrial biochemical process that could be improved by a more efficient use of the immobilized enzyme.

3. The immobilized enzyme can be used therapeutically.

These three motivations lead to very different ways of thinking about

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62. Reedy, C.C., "Assays used to Determine the Presence and Activity of the Enzyme Heparinase," S.B. Thesis, MIT, June, 1980.

immobilized enzymes. The first case leads one to consider immobilized enzyme reactions where the substrate concentration is low, where there are competing or sequential enzymes co-immobilized, or where there are enzyme inhibitors present. The second case is mostly concerned with industrial processes run at high substrate concentrations with high purity enzyme and substrate. The third case assumes a low substrate concentration, a pure enzyme, and sometimes the presence of inhibitors. These three different cases of immobilized enzymes have some kinetic aspects in common, but one must be careful when generalizing across their boundaries, as different assumptions are made in each case.

VARIATIONS FROM FREE ENZYME KINETICS.

There are four main reasons why an immobilized enzyme may exhibit reaction kinetics different from the same enzyme in solution:

1. A conformational change in the enzyme during immobilization may give the enzyme a different activity, as the activity of an enzyme is very dependent on its conformation. These changes are often severe enough to cause a complete loss of activity.

2. Since the enzyme is bound to a solid support, the microenvironment surrounding the enzyme may be different from the environment of the bulk solution. Studies of enzyme reactions in different solvents have shown that environmental effects can be very profound.[63 64 65] The actual reaction

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63. Barnard, M.L., and K.J. Laidler, "Solvent Effects in the a-Chymotrypsin Hydrocinnamic Ester System," J. Amer. Chem. Soc., 74, 6099-6101 (1952).

64. Laidler, K.J. and M.C. Ethier, "Molecular Kinetics of Muscle Adenosin Triphosphatase: Solvent and Structural Effects" Arch. Biochem. Biophys. 44, 338-345 (1953).

65. Findlay, D., A. P. Mathias, and B.R. Rabin, "The Active Site and Mechanism of Action of Bovine Pancreatic Ribonuclease: Charge types at the Active Centre," Biochem. J., 85, 139-144 (1963).

between enzyme and substrate is occurring in this microenvironment. The kinetic behaviour of the enzyme will therefore be determined by conditions at the surface of or inside the solid support, and may not be the behaviour one would expect from bulk solution conditions.

3. There is some partitioning of the substrate between the solution and the support, due to either polar or electrostatic effects. Thus a polar substrate is more likely to enter a support with many polar groups and a non-polar substrate is more likely to enter a support with many non-polar groups. If both the substrate and the support are electrically charged, this can effect the entry of the substrate into the support.[66]

4. Also, the rate of reaction may be controlled by external or internal mass transfer, or some combination. External mass transfer is the process by which the substrate diffuses from the bulk solution to the surface where the enzyme has been immobilized. Internal mass transfer is the net movement of the substrate through pores from the surface to the interior of the support. These diffusion effects can influence the concentration of substrate and products both at the surface and inside the support.[67] The effects of internal and external mass transfer can be considered independently of the partitioning if the value for the effective diffusivity in the pores of the support takes takes the partitioning into account.[68] Diffusion effects can also alter the apparent mode of action of an enzyme.[69]

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66. Goldstein, L., Y. Levin, and E. Katchalski, "A Water-insoluble Polyanionic Derivative of Trypsin: Effect of the Polyelectrolyte Carrier on the Kinetic Behavior of the Bound Trypsin," Biochem., 3, 1913-1919 (1964).

67. Sundaram, P.V., A. Tweedale, and K.J. Laidler, "Kinetic Laws for Solid Supported Enzymes," Can. J. Chem., 48, 1498-1504 (1970).

68. ibid.

A reactor using immobilized heparinase to continuously remove heparin from the bloodstream has been proposed[70] and tested in canine and human blood.[71] The reactor used in that study will be analyzed using theoretical models for immobilized enzyme kinetics to determine whether the rate of reaction between heparin and the immobilized heparinase is controlled by either the internal or external rates of diffusion or the intrinsic kinetics of the reaction. Then in the DISCUSSION section, I will make some suggestions to improve the efficiency of the reactor based on this analysis.

MODELS FOR IMMOBILIZED ENZYME KINETICS.

Two models for the reaction of immobilized heparinase and heparin will be considered. The first will assume that the enzyme is evenly distributed throughout the beads, with reaction taking place principally inside the beads. The second model assumes that the enzyme is bound primarily at the surface of the bead, and that the substrate does not penetrate the bead. These two models are called the internal reaction model and the external reaction model respectively.

THE EXTERNAL REACTION MODEL.

There are two parts to the external reaction model: mass transfer of the

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69. Lee, D.D., G.K. Lee, P.J. Reilly, and Y.Y. Lee, "Effect of Pore Diffusion Limitation on Dextrin Hydrolysis by Immobilized Glucoamylase," Biotech. Bioeng., 22, 1-17 (1980).

70. Langer, R., R.J. Linhardt, P.M. Galliher, M.M. Flanagan, C.L. Cooney, and M.D. Klein, "A System for Heparin Removal," in Biomaterials: Interfacial Phenomena and Applications, S. Cooper, A. Hoffman, N. Peppas, B Rattner, eds., Washington, D.C., American Chemical Society, 493-509 (1982).

71. Langer, R., R.J. Linhardt, S. Hoffberg, A.K. Larsen, C.L. Cooney, D. Tapper, and M. Klein, "An Enzymatic System for Removing Heparin in Extracorporeal Therapy," Science, 217, 261-263 (1982).

substrate from the bulk solution into the surface of the bead and reaction at the surface of the bead. Electrostatic effects are considered negligible for this model. The thickness of the Gouey diffuse double layer, the layer above the surface of the support where the electrostatic effects are considered significant, is on the order of 10 Å.[72] This is less than the diameter of the enzyme. Therefore the reaction at the active site of the enzyme should be unaffected by the charge of the support. The equations governing the external mass transfer rate (V_{mt}) is:

$$V_{mt} = \beta (C_{inf} - C_{sur}) \qquad \text{Eq. 3-1}$$

where C_{inf} is the concentration of substrate in bulk solution (mole/m^3) , C_{sur} is the concentration of substrate at the surface of the bead (mole/m^3), and β is a mass transfer coefficient (m/s) found by the correlation:[73]

$$\beta = \frac{D}{2\,r} \left(2 + .6\ Re^{.5}\ Sc^{1/3} \right) \qquad \text{Eq. 3-2}$$

where D is the diffusivity of the substrate in the fluid (m^2/s), r is the radius of the bead (m), Re is the Reynolds number[74] (dimensionless), and Sc is

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72. Shuler, M.L., R. Aris, and H.M. Tsuchiya, "Diffusive and Electrostatic Effects with Immobilized Enzymes," J. Theor. Biol., 35, 67-76 (1972).

73. Bird, R.B., W.E. Stewart, and E.N. Lightfoot, Transport Phenomena. New York, John Wiley & Sons, 647 (1960).

the Schmitt number[75] (dimensionless). The equations governing the reaction at the surface (assuming that the concentration of substrate at the surface is much less than K_m) are:

$$V_{rxn} = k_{cat}\ E\ C_{sur} / K_m \qquad \text{Eq. 3-3}$$

where k_{cat} is the turnover number of the enzyme (s^{-1}), E is the concentration of enzyme ($mole/m^2$), C_{sur} is the concentration of substrate at the surface of the bead ($mole/m^3$), and K_m is the Michaelis constant for the immobilized enzyme ($mole/m^3$). At steady state, $V_{mt} = V_{rxn}$, and equations 3-1 and 3-3 can be set equal and solved for C_{sur}. Back substitution of C_{sur} then gives the rate of the reaction. The above equations were used with constants taken from the in vivo experiments with dogs (see Appendix C). The rate of reaction was found to be 1.7×10^{-8} mole/s as compared to the observed rate of reaction (based on 4 units/ml heparin concentration, 50 ml/min flow rate, and 90% conversion) of 1.5×10^{-9} mole/s.

THE INTERNAL REACTION MODEL.

There are three parts to the internal reaction model: diffusion from the bulk solution to the surface of the bead, a partitioning of the substrate between the solution and the interior of the bead caused by electrostatic

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74. The Reynolds number is defined here as ($D\ v\ \rho / \eta$), where D is the diameter of the bead, v is the velocity of the fluid, ρ is the fluid density and η is the fluid viscosity. Any consistent group of units may be used so long as the Reynolds number is dimensionless.

75. The Schmitt number is defined here as ($\eta / \rho\ D_s$) where η is the fluid viscosity, ρ is the fluid density, and D_s is the diffusivity of the substrate through the fluid.

repulsion (or attraction) between the substrate and the bead, and reaction inside the bead. The equations governing external mass transfer for the internal reaction model are the same as for the external reaction model. The equation governing the partitioning of the substrate between the solution and the interior of the bead for charged beads and substrates is:[76]

$$C_{s,int} = C_{s,ext} \exp(- \lambda / 2.303) \qquad \text{Eq. 3-4}$$

where $C_{s,int}$ is the inside surface concentration of substrate (mole/m^3), $C_{s,ext}$ is the outside surface concentration of substrate, and λ is a partition coefficient (dimensionless) described by:[77]

$$\lambda = \frac{z F \psi}{R T} \qquad \text{Eq. 3-5}$$

where z is the charge on the substrate, F is Faraday's constant, ψ is the potential of the bead at its surface, R is the gas constant, and T is the temperature. ψ can be measured experimentally by noting the shift in optimal pH upon immobilization.[78] The partition coefficient for hydrogen can then be

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76. Hamilton, B.K., L.J. Stockmeyer, and C.K. Colton, "Comments on Diffusive and Electrostatic Effects with Immobilized Enzymes," J. Theor. Biol., 41, 547-560 (1973).

77. Shuler, M.L., R. Aris, and H.M. Tsuchiya, "Diffusive and Electrostatic Effects with Immobilized Enzymes," J. Theor. Biol., 35, 67-76 (1972).

78. Goldstein, L., Y. Levin, and E. Katchalski, "A Water-insoluble Polyanionic Derivative of Trypsin: Effect of the Polyelectrolyte Carrier on the Kinetic Behavior of the Bound Trypsin," Biochem., 3, 1913-1919 (1964).

where k_{cat} is the turnover number of the enzyme (s^{-1}), E is the concentration of enzyme in the pellet ($mole/m^3$), K_m is the Michaelis constant of the enzyme ($mole/m^3$),and D_e is the effective diffusivity of the substrate in the pores of the bead. D_e is usually expressed as the diffusivity of the substrate in the solvent divided by a factor τ, the tortuosity. The boundary conditions are obtained by applying the concept of pellet symmetry at the center of the bead to show that the concentration gradient is zero there, and by applying the concept of continuity at the inside surface of the bead, since the concentration there can theoretically be found by solving the equations for external mass transport and partitioning.

This type of reaction-diffusion combination is usually analyzed in terms of an effectiveness factor, η, taken as the ratio between the actual rate of reaction for the entire pellet and the rate evaluated at the surface conditions:

$$V_{pellet} = \eta V_{surface} \qquad \text{Eq. 3-8}$$

A useful parameter for this analysis is Φ_s, the Theile modulus for a spherical pellet:

$$\Phi_s = \frac{r_s}{3} \left(\frac{k_{cat}\, E}{K_m\, D_e} \right)^{1/2} \qquad \text{Eq. 3-9}$$

Solving Eqs. 3-7 in terms of the Theile modulus yields and equation for the effectiveness factor:[83]

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83. Smith, J.M., Chemical Engineering Kinetics, 3rd ed., New York, McGraw-Hill, p. 479 (1981).

$$\eta = \frac{1}{\Phi_s}\left(\frac{1}{\tanh(3\Phi_s)} - \frac{1}{3\Phi_s}\right) \qquad \text{Eq. 3-10}$$

Substituting Eq. 3-10 into Eq. 3-8,

$$V_{pellet} = \frac{1}{\Phi_s}\left(\frac{1}{\tanh(3\Phi_s)} - \frac{1}{3\Phi_s}\right)\frac{k_{cat}\,E}{K_m}\,C_{s,int} \qquad \text{Eq. 3-11}$$

Again, at steady state, the rate of mass transfer given by Eq. 3-1 must equal the rate of reaction given by Eq. 3-11. The overall rate of reaction is thus easily solved for as above. The detailed calculations are shown in Appendix B. Table 3-1 shows the results of the internal reaction model for various values of z, the charge on the heparin molecule, and τ, the tortuosity of the pores in the Sepharose bead. The value for the tortuosity supplied by Sigma Chemical company for their Sepharose beads is 3, measured by protein uptake from solution.[84] The capacity of the gel is 10 mg protein per gram of gel for enzymes such as heparinase with a molecular weight of about 50,000.[85] The reactor used 1 mg protein per gram of gel in the immobilization,[86] implying

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84. Pharmacia Fine Chemicals Company, Affinity Chromatography: Principles and Methods. Pharmacia Fine Chemicals, Uppsala, Sweden.

85. ibid.

86. Langer, R., R.J. Linhardt, S. Hoffberg, A.K. Larsen, C.L. Cooney, D. Tapper, and M. Klein, "An Enzymatic System for Removing Heparin in Extracorporeal Therapy," Science, 217, 261-263 (1982).

Table 3-1. Reaction Rates Predicted by Internal Diffusion Model

Z	tortuosity*	Reaction Rate (mol/s)
-47.4	1 - 10	1.33×10^{-29}
-40	1 - 10	4.91×10^{-27}
-30	1 - 10	1.44×10^{-23}
-20	1 - 10	4.23×10^{-20}
-10	1 - 10	1.24×10^{-16}
0	1 - 10	3.64×10^{-13}
10	1 - 10	1.03×10^{-9}

* Values for the reaction rate are insensitive in the third decimal place to changes in the tortuosity in this range.

that only 10% of the linking sites were used in the immobilization. The value of -47.4 for z is based on 2.37 sulphate moieties per disaccharide in the heparin chain[87], and an average of 20 disaccharides per molecule, each sulphate bearing a single negative charge at neutral pH. Notice that the predicted rate of reaction for this instance is almost 20 orders of magnitude below the observed value. Only by reducing both the charge and the tortuosity severely does this model approach the observed rate of reaction.

No further experiments are necessary to conclude that the reaction probably occurs primarily on the surface of the Sepharose beads. The 91% retention of heparinase activity can be explained by the very low utilization of CNBr sites on the Sepharose 4B support.

INDEPENDENCE OF THE CLEAVABLE ALPHA LINKAGES.

Heparin structure can be explored using heparinase. This study is intended to determine if the heparinase-cleavable alpha linkages of heparin are randomly distributed throughout the heparin molecule. First, models for the distribution of the heparinase-cleavable sites are proposed. These models all imply a final distribution of product sizes. Then these implied product distributions are compared with the experimental final product distribution to evaluate the accuracy of any given model for the distribution of heparinase-cleavable sites in heparin.

All of the proposed models depend on experimentally measured parameters. For the equilibrium distribution, the initial molecular weight distribution of

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87. Rosenberg, R.D., G. Armand, and L. Lam, "Structure-Function Relationships of Heparin Species," Biochemistry, 75, 3065-3069 (1978).

heparin and the percent of the alpha linkages which are heparinase-cleavable are the only experimental parameters required to predict the final product distribution. To model the distribution of products through time, the affinity of heparinase for different sized chains of heparin and for different sites in the heparin chain must also be measured and integrated into the models.

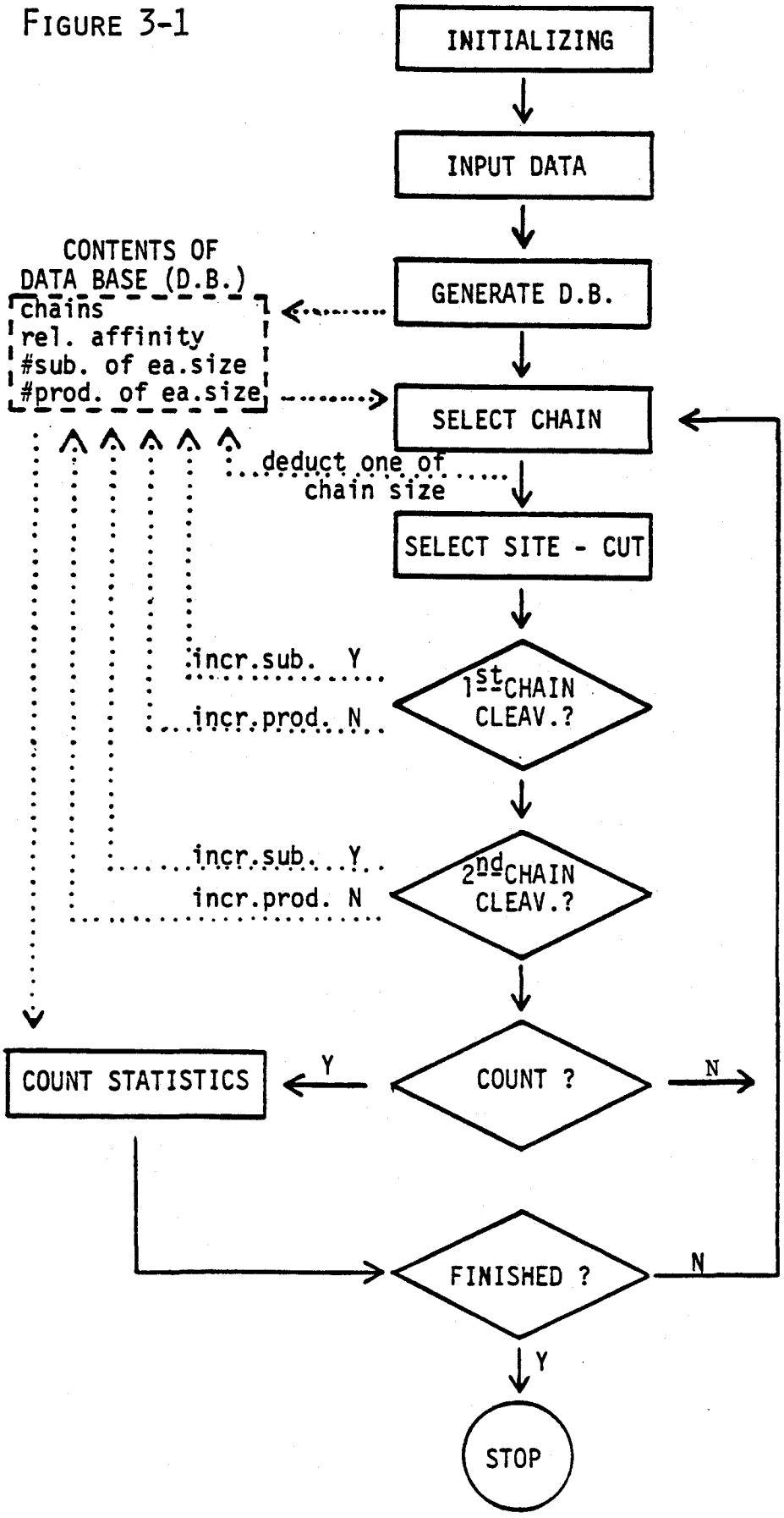
In general, the models were constructed from modules. Each module took the program through a particular step in the process flowchart. By keeping the program modular, particular steps of the program could be changed with a minimum of effort. For example, the initial data base of chains could be constructed using either an assumption of total independence of the heparinase-cleavable alpha linkages or the assumptions of diad analysis (see below), and the only section of the program that would need to be changed is the section of the program concerning the construction of the initial database.

EXPLANATION OF THE COMPUTER MODEL.

The overall flowchart of the heparinase model is given in Figure 3-1. Appendix A contains the complete coded program and cross-reference tables. The program begins by setting up formats for the data, inputs, and outputs. User defined functions are defined in the initialization step as well. These functions are generally used throughout many of the modules, and are kept in this initialization step to save time and space. The next section of the program takes the initial data: 1) the initial molecular weight distribution of the heparin, 2) the relative affinity of heparinase for different sizes of

FLOW CHART OF THE COMPUTER SIMULATION OF HEPARINASE ACTION

FIGURE 3-1



heparin chains (necessary for the kinetic model only), and 3) the percentage of alpha linkages which are heparinase cleavable. The third section of the program takes this data and constructs an initial database according to a specific set of rules. In Appendix A, the module used in the third section assumes that cleavability of any alpha linkage is independent of the cleavability of any other alpha linkage. Other possible modules include the use of diad or triad analyses,[88] or a template insertion module. Diad (or triad) analysis constructs copolymer chains based on the probability of each monomer being added to the chain, given the structure of the preceding two (or three) monomers and the probability of each initial dimer (or trimer). The template insertion module is compatible with the program module that assumes independent probabilities for the extension of chains, and is also included in the sample program in Appendix A. Template insertion is based on an assumption that a particular sequence of cleavable/uncleavable sites is favoured over other sequences of the same length.[89] Template insertion is accomplished in this module by first creating a database by any other section three module, and then mutating that database by inserting the specified sequence at random. The exact number of templates to be inserted is computed by a complex algorithm described below under the heading 'SENSITIVITY OF THE COMPUTER MODEL'.

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88. Billmeyer, F.W., Textbook of Polymer Science, 2nd ed., New York, Wiley Interscience, 328-354 (1971).

89. Biologically, this may be thought of as a particular biosynthetic mechanism which causes a particular sequence of sugars to occur more frequently.

Sections 4 through 7 of the model are concerned with the actual cleavage of the chains. Section 4 selects a chain to be cleaved. In the model given in Appendix A, this selection is done assuming each chain to have an equal chance of being cleaved, independent of length. Other modules that could be inserted here could perform the selection of chains weighted to the weight of the chain. Once the chain is selected it is retrieved from the database and then deleted. The selected chain is inspected by the program to determine the number of cleavable sites it contains. One of these is selected at random, as the enzyme is known to have a random endolytic mode of action.[90] There is nothing to prevent the program from using a weighted method for choosing a site. The chain is then split into two pieces, eliminating the selected cleavable linkage. Each chain carries a string identifying whether it carries an original right or left (reducing end or non-reducing end) end group. Thus the model automatically keeps track of any artifacts caused by the original end groups on the heparin molecules. Each of the two newly created chains is then quickly tested to see if it still contains any cleavable sites. If so, the chain is placed in the database on an equal footing with all other chains. Alternately, these chains could be placed in a section of the database that would have a higher or lower probability of being cleaved. If the new chain does not contain any cleavable sites, information about that chain is recorded, such as the length of the chain, what endgroups it contained, and the chain is then discarded. One cut is recorded, ending the active part of the cutting loop.

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90. Linhardt, R.J., G.L. Fitzgerald, C.L. Cooney, and R. Langer, "Mode of Action of Heparin Lyase on Heparin," Biochimica et Biophysica Acta, 702, 197-203 (1982).

During database generation, the number of cleavable sites was counted. The "Count?" module sends the program to a statistics routine any time a preset percentage of the degradation has occurred. In the program in Appendix A, the "Count?" module is set to record the condition of the database every 5% of the way through the degradation. The statistics about the database that are recorded include the molecular weight (length) distribution of the chains, the number, weight, and Z average molecular weights, and the molecular weight distributions for chains having original right and left ends, and with and without cleavable sites. This information can be used to analyze, for instance, what percentage of the disaccharides in the final distribution will have at either their reducing or non-reducing ends the original heparin end groups, and what percentage will reflect the structure of a heparinase-cleavable site. The model is considered complete when all of he cleavable sites have been cut. A final statistical count is done on the final distribution of chains.

SENSITIVITY OF THE COMPUTER MODEL.

This investigation is designed to test the validity of the assumption that the heparinase-cleavable sites in heparin are distributed throughout the heparin molecular chains in a random independent manner. Random implies that each alpha linkage is added to the chain one at a time, that there are no blocks of prefabricated chains to be added to the main heparin chain. Independent implies that each alpha linkage or block of alpha linkages is added to the heparin chain with a probability that does not depend on the structure of any other part of the heparin chain. To obtain a random, independent ensemble of chains for use in the computer model of heparin degradation, all that is necessary is to specify the number of chains of each

size in the ensemble, and throw a die weighted by the percent of uncleavable sites for each alpha linkage in that ensemble of chains. Ensembles of chains generated in this manner will produce a particular distribution of products when all of the cleavable sites have been cleaved. To test the sensitivity of the final product distribution to the assumptions made about the distribution of cleavable alpha linkages, small amounts of dependence and non-randomness were added to the mechanism for generating the original ensemble of heparin chains.

To test how sensitive the final product distribution is to small variations in the distribution of cleavable alpha linkages, the original database of chains was constructed using a variety of deviations from a random, independent assortment of cleavable and uncleavable alpha linkages. These deviant methods are known as diad analysis,[91] triad analysis,[92] and template insertion. Diad and triad analyses introduce dependence into the initial distribution of heparinase-cleavable sites by using conditional probabilities to extend length of the chain. Template insertion introduces non-randomness into the initial distribution by ensuring that a particular sequence of cleavable and uncleavable alpha linkages occurs more often than it would occur in the random, independent model.

DIAD ANALYSIS.

Diad analysis begins by selecting an initial dimer for the chain. If 1 is used to signify a cleavable site, and 0 an uncleavable site, then the four

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91. Billmeyer, F.W., Textbook of Polymer Science, 2nd ed., New York, Wiley Interscience, 328-354 (1971).

92. ibid.

possible initial dimers (considering disaccharides as the monomer for this analysis only) are 00, 01, 10, and 11. The diad analysis model can be considered random if the probability for each of these initial dimers is determined by multiplying the appropriate constants for "the probability of a cleavable site" or "the probability of an uncleavable site." The initial dimer has the same overall composition under diad analysis as under the random, independent model. The third site, however, is chosen by a probability distribution that is dependent on the structure of the first two sites. Subsequent sites are also chosen based on the structure of the two previous sites. Diad analysis is a model that assumes some dependence within a random structure. This dependence can be used to make either alternating or repeating sequences more prevalent than they would be in an independent construction of the chains. Therefore there is less information in the structure of the chains thus constructed. Chains constructed by a diad analysis method would produce a final product distribution different than that produced by a random, independent method, but the degree of difference would depend on the nature of the ensemble of chains and the degree of dependence used in the analysis.

TRIAD ANALYSIS.

Triad analysis works in basically the same was as diad analysis. The major differences are that the initial trimer is selected at random (rather than the initial dimer) and that the cleavability of each alpha linkage is dependent on the cleavability of the previous 3 (rather than 2) alpha linkages. Again, setting the probabilities of a cleavable or uncleavable site given each of the eight possible preceding trimers will favour some

constructions over others, depending on the values used. Again the final product distribution obtained by complete degradation of an ensemble of chains constructed with triad analysis would be expected to be different from that obtained from a random, independent ensemble of chains.

SHORTCOMINGS OF DIAD AND TRIAD ANALYSIS.

While both diad and triad analysis can test the sensitivity of the final product distribution to the distribution of cleavable sites, it is difficult to quantitate the actual amount of difference between the ensemble of chains generated by a random independent method of chain construction and either random dependent method. Even more difficult with these methods is controlling the percentage of cleavable sites actually used in construction of the original ensemble of chains. Only after many attempts with various algorithms for controlling both the percent of cleavable sites and the amount dependence in the model did I discover in a classical work in information theory by Claude Shannon that this type of dual control was computationally very difficult. Even assuming an efficient algorithm could be devised, the computation is likely to take too much computer time to be feasible.[93] No attempt was made to control the percent of cleavable sites for either the diad or triad analysis methods of chain construction. Consequently, the product distributions predicted using these methods of chain construction varied greatly from trial to trial.

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93. Shannon, C.E., and W. Weaver, The Mathematical Theory of Communication, Urbana, Ill., The University of Illinois Press (1949).

Shannon's book suggests another method of approach. His work in information theory provides a method for determining the amount of randomness (or informational entropy, as he calls it) present in a series of signals, with applications toward encryption and decryption of messages. Thus, if some sequence of signals occurred more (or less) often than would be expected from a random, independent assortment of signals, this would lower the amount of informational entropy in the message, and indicate the presence of some "signal".

TEMPLATE INSERTION.

The template insertion method of generating non-random independent ensembles of chains uses this principle. A sequence of cleavable and uncleavable sites (e.g. '101') is chosen. The number of these sequences expected in the random, independent ensemble of chains is predicted (how this is done is explained below). Then an additional amount (e.g. 2% of the expected number) of this template is inserted into the ensemble of chains at random. In this way a small signal of known size is placed in the structure of the chains. The template is added rather than removed from the ensemble of chains (either would generate a signal) because inserting 2% more of a template is computationally easier than searching the ensemble of chains for each occurence of a template and giving each occurrence a 2% chance of being changed.

PREDICTING TEMPLATE OCCURRENCE: OVERLAPS ALLOWED.

The remaining challenge to implementing the template insertion method was accurately predicting the number of times a particular sequence would be expected to occur in a given random, independent ensemble of chains. If the

template is allowed to overlap, (e.g. for the template '101', the sequence '10101' would count as 2 occurrences of the template) the method of prediction is straightforward. For a chain of length M, and a template of length T containing U uncleavable sites, with P being the overall probability of an uncleavable site (equal to the percentage of uncleavable sites), the expected number of templates in the chain is:

$$\text{Expected \# of templates} = (M-T+1) \times P^{U} \times (1-P)^{(T-U)} \qquad \text{Eq. 3-11}$$

The term (M-T+1) is the number of places in the chain that a template of that length could be found. The other two terms define the probability of the particular template being found relative to all other templates of that length. This equation can be used to find the expected number of templates (overlapping allowed) in a given ensemble of chains given the number of chains N_M of each length M in the ensemble. The formal expression is:

$$\sum_{i=1}^{M} N_M \times \text{Expected \# of templates in chain of length M} \qquad \text{Eq. 3-12}$$

PREDICTING TEMPLATE OCCURRENCE: NO OVERLAPS ALLOWED.

Computing the expected number of occurrences of a particular template in a chain without counting overlapping occurrences (e.g. for the template '101', the sequence '10101' would count as only 1 occurrence of the template) is a much more complicated problem. The algorithm I am describing is offered here without proof, with only the statement that it seems to work. The first step

is to find the set of character sequences containing fewer characters that the original template that, when added to the right-hand end of the chain, will produce a sequence containing two or more overlapping occurrences of the template. This set is called the main set. If this set is null, then the formula used for overlapping templates can be used, as there is no way that the particular template can form overlapping sequences. If this set contains character sequences which are multiples of one another (e.g. '01', '0101', and '010101'), the largest and smallest character sequences are kept as a separate set (called the multiple set for lack of a better name) and all members of that family are deleted from the main set.

The expected number of templates in the ensemble of chains is then computed by the following algorithm.

1. Find the expected number of templates in the ensemble as if overlapping was allowed.

2. Add each character sequence in the main set (including the <u>smaller</u> character sequence in the multiple set) to the template in turn, and find the expected number of each "template plus 1 character sequence" allowing overlaps, and <u>subtract</u> each value from the expected number found in Step 1. Any "template plus 1 character sequence" that is larger than the largest chain in the ensemble can be discarded before going to the next step.

3. To each "template plus 1 character sequence", add each character sequence in the main set (including the <u>larger</u> character sequence in the multiple set). Find the expected number of each "template plus 2 character sequences" allowing overlaps, and <u>add</u> each value to the expected number found in Step 1. Any "template plus 2 character sequences" that is larger than the largest chain in the ensemble can be

discarded before going to the next step.

4. Steps 2 and 3 are repeated until all "template plus N character sequences" are larger than the largest chain in the ensemble. The sum obtained after all the adding and subtracting is done is the expected number of times that the specified template will occur in the ensemble of chains, not counting the instances where it overlaps itself.

Although this algorithm appears to be quite complex, it is actually quite simple to implement in most computer languages capable of string arithmetic. In the model given in Appendix A, the operator has a choice between the overlapping and non-overlapping strategies for each template.

4. MATERIALS AND METHODS

ASSAYS.

The principle assays used in this investigation were: the Azure A assay for measuring heparin concentration, the UV 232nm assay for measuring the concentration of heparin degradation products, and the Biorad protein assay for protein.

THE AZURE A ASSAY.

Jacques[94] has proposed that Azure A dye molecules dimerize in the presence of heparin resulting in a decrease in the pi delocalization. This is observed as a shift in the absorbance maxima of the dye molecules from λ_{max} = 620nm to λ_{max} = 520nm. Since heparinase cleaves the alpha linkage of heparin, its action causes chain shortening resulting in less metachromasia. The presence of heparin or heparin-like polysaccharides of hexasaccharide length or longer[95] can be measured reproducibly at levels of 1 to 10 mg/ml in crude and 1 to 10 ug/ml in purified preparations of heparinase.[96] The concentration of these polysaccharides is measured by diluting the reaction mixture into this range with 0.02 g/l Azure A dye and measuring the optical absorbance at 620nm. Before the heparin concentration can be known, standards of known concentration must be assayed and plotted on a standard curve. The measurement at 620nm measures the appearance of the blue hue. Absorbance at

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94. Jacques, L.B., "Heparin: An Old Drug with a New Paradigm," Science, 206, 528-533 (1979).

95. Dietrich, C.D., "Novel Heparin Degradation Products," Biochem. J., 108, 647-654 (1968).

96. Reedy, C.C., "Assays used to Determine the Presence and Activity of the Enzyme Heparinase," S.B. Thesis, MIT, June, 1980.

530nm can be read to follow the purple hue, but this has been found to be a less accurate procedure.[97]

THE UV 232 NM ASSAY.

The molar amount of products was assayed by absorbance at UV 232nm. Because heparinase is an alpha 1,4-eliminase, it cleaves heparin leaving an α,β unsaturated endgroup. This chromophore has a maximum absorbance at approximately 232nm and a molar absorption of about 5.5×10^{-3} Optical Density units per mole.[98] The concentration of heparin products is followed by diluting the reaction mixture to about 0.1 mg/ml of product in distilled water and measuring the optical absorbance. This assay method can be used only for purified enzyme preparations. In crude enzyme preparations the high concentration of protein lowers the assay's sensitivity and contaminating enzymes, especially glycuronidases, catalyse the loss of the chromophore. This assay is recommended for its simplicity, enabling the experimenter to run many reactions simultaneously.

Glycuronidase was assayed by measuring the decrease in absorbance at UV 232nm of fully digested heparin. Heparin was fully degraded by the heparinase preparation and the absorbance recorded. More of that preparation of heparinase was added to the reaction mixture and the absorbance assayed at UV 232nm at intervals over the following 36 hours.

Protein was assayed by the Biorad Protein Assay.[99]

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97. Reedy, C., "Assays used to Determine the Presence and Activity of the Enzyme Heparinase," S.B. Thesis, MIT, June, 1980.

98. Linker, A. and P. Hovingh, "Isolation and Characterization of Oligosaccharides Obtained from Heparin by the Action of Heparinase," Biochemistry, 11, 563-568 (1972).

99. Bradford, M.M., "A Rapid and Sensitive Method for the Quantization of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding," Anal. Biochem., 72, 248-254 (1976).

GEL PERMEATION CHROMATOGRAPHY.

Gel permeation chromatography was used to separate the original heparin by molecular weight and also to separate the products of the heparin-heparinase reaction.

THE MOLECULAR WEIGHT FRACTIONATION OF HEPARIN.

Heparin is porcine heparin purchased from the Sigma Chemical Company (from intestinal mucosa, H-7005, grade II, sodium salt, 151 units/mg, Lot 110F-0323). Sephadex^{R} G-75 was also obtained from the Sigma Chemical Company Stock #G75-120, Lot 69C-0056). The heparin was fractionated on a 50 cm Sephadex G75 column 1.5 cm in diameter. The void volume of the column was 26.1 ml as measured by the elution of blue dextran. 50 mg of heparin were loaded onto this column and run using 1 Molar NaCl at a flow rate of 0.16 ml/min. The heparin was collected into fractions of approximately .5 ml. A 10 ul aliquot of each fraction was assayed by Azure A assay to determine the elution profile of heparin. The fractions from fifteen 50mg runs were combined into 5 molecular weight fractions containing equal weights of heparin. Each of the five fractions was then dialysed in 1000 MW dialysis bags (Spectrapor Membrane Tubing, #132636, Spectrum Medical Industries, Los Angeles) against distilled water. The dialysis bags were then opened and the solutions freeze dried (Labconco Freeze-drier Model 75040, Labconco, Inc., Kansas City, Mo.) and weighed. Each of the molecular weight fractions was then rerun on the G75 column to get an accurate molecular weight determination. Each of the five fractions was recovered, dialysed, freeze-dried, and weighed.

MOLECULAR WEIGHT STANDARDS FOR G75.

Polyethylene Glycols of molecular weights 3750 and 5700 were run on the column in 1 Molar NaCl as molecular weight standards. The polyethylene glycol was detected spectrophotometrically at 207 nm using the 1 Molar NaCl as a blank.

SEPARATION OF FINAL PRODUCTS ON FRACTOGEL.

Following the degradation of the heparin, the final degradation products were separated on a 1.5 meter fractogel column having a diameter of 1.5 cm and a void volume of 66.0 ml by elution of blue dextran. The products were eluted with 1 Molar NaCl at a flow rate of 100 ul/min. Fractogel {trademark} TSK HW-40 (S) was purchased from MCB Manufacturing Chemists, Inc., Cat. No. 14983-6. The fractogel was obtained pre Swollen, containing 0.02% NaN_3.

REAGENTS.

Heparinase (E.C. 4.2.2.7) was obtained fermentatively from Flavobacterium heparinum and was purified by Cynthia Zannetos using hydroxylapatite (S.A. = 140 mg Heparin degraded/mg protein-hr, .22 mg/ml protein, designated HA)[100] [101] and cellulose phosphate purified (spec. act. = 2172, .001 mg/ml protein, designated CP) as in C. Zannetos' thesis.[102] Azure A was purchased from the Fisher Scientific Co. of Pittsburg, Pa., #A-9770, Lot 774063. Blue Dextran

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100.Langer, R., R.J. Linhardt, M. Klein, P.M. Galliher, C.L. Cooney, and M.M. Flanagan, "A System for Heparin Removal", in Advances in Chemistry,(ed. by S. Cooper, A. Hoffman, N. Peppas, and B. Rattner), 1982.

101.Linhardt, R.J., R. Langer, C.L. Cooney, P.M. Galliher, M.M. Flanagan, and S.M. Hoffberg, "Deheparinization using Immobilized Microbial Heparinase", in Proceedings of the Second World Congress of Chemical Engineering, Vol. I. Montreal.

was purchased from Pharmacia Fine Chemicals Co., Lot C819. Polyethylene glycols were purchased from Polysciences, Inc., Warrington, PA (Cat. No. 15648 MW 3750, Mw/Mn = 1.1, Cat. No. 15649 MW 5700, Mw/Mn = 1.10). All inorganics were reagent grade or better.

Two buffer systems for the enzymatic reaction were used in this study. They have been designated as "New Assay Mix" (NAM) and "Old Assay Mix" (OAM). They are defined as follows: New assay mix is 0.1 M Phosphoric, Boric, and Acetic acids and may have its pH adjusted as desired with 10N NaOH. Old Assay mix is 0.25 M in Sodium Acetate and 2.5×10^{-3} M in calcium Acetate adjusted to pH 7.0 with 10 N NaOH.

OTHER EQUIPMENT.

All Spectrophotometric measurements were made with a Gilford model 3723 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

COMPUTER MODELING.

Modeling was done on a Digital Equipment Corporation VAX 11/780 running under VAX/VMS Version 2.5 in Digital BASIC Language, Version 3.0. Fully commented copies of the models are in Appendix A.

102.Zannetos, C., "The Purification of Heparinase from Flavobacterium Heparinum," S.M. Thesis, MIT, March, 1983.

5. RESULTS

MOLECULAR WEIGHT FRACTIONATION OF HEPARIN.

Fifty mg of heparin was dissolved into 0.5 ml of a 1 M NaCl solution and loaded onto the G75 column and eluted with 1 M NaCl at a flow rate of 160 ul/min. The high salt concentration is necessary to prevent the heparin from sticking to the Sepharose.[103] Initial fractions were collected and assayed for heparin by the Azure A assay. An example of the elution profile is given in Figure 5-1. Individual elution profiles varied by as much as 0.1 mg/ml at the peak concentration and the peak varied by as much as 3 ml of elution volume. The fractions were combined to make five fractions having different molecular weights, each fraction containing approximately 10 mg of heparin. After five 50 mg samples of heparin had been fractionated, corresponding molecular weight fractions were combined. Each fraction was dialysed in 1000 MW cutoff dialysis bags against distilled water and then freeze-dried for two days at 10-50 millitorr pressure. The white powder obtained was then redissolved in 0.5 ml of 1 M NaCl and reapplied to the G75 column as before. The elution profiles of the fractions are given in Figure 5-2.

STANDARDIZATION OF THE G75 COLUMN.

To estimate the molecular weight of the fractions, the elution of molecular weight standards from the G75 column was observed. Polyethylene glycols were chosen over protein standards, as heparin tends to be rod-like in solution, like polyethylene glycol and unlike globular proteins. Ten mg of

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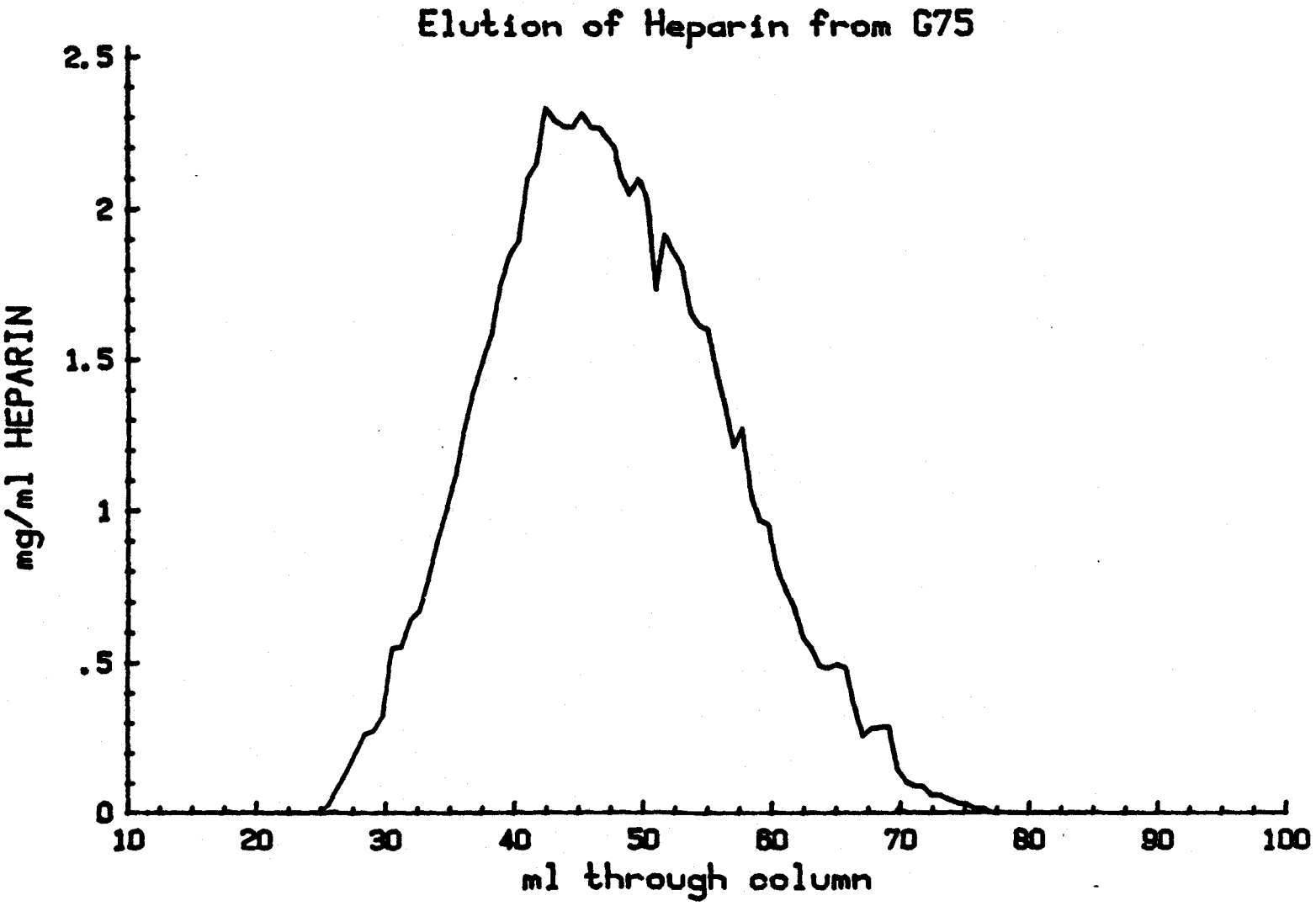
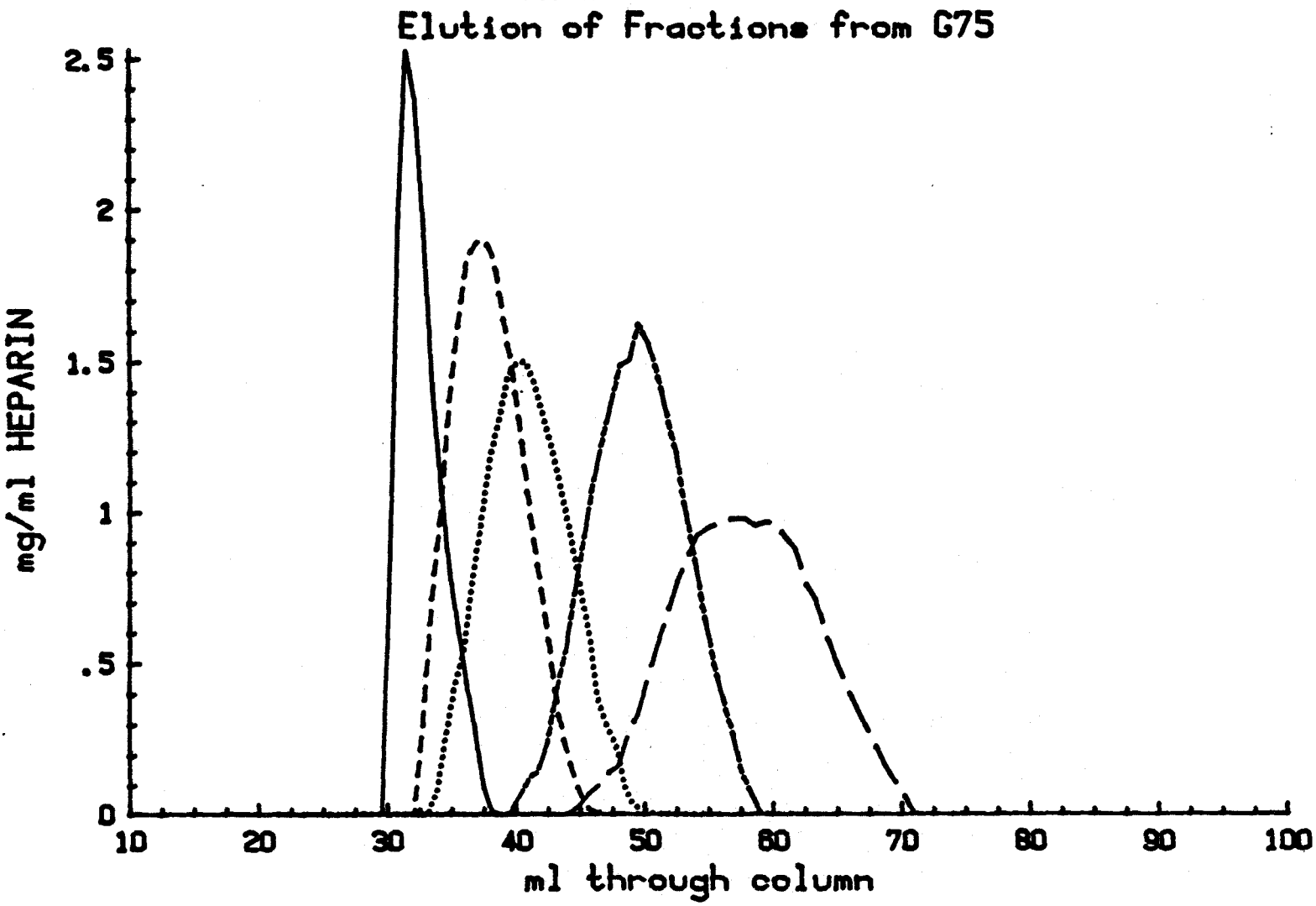
103.Liberti, P.A., and S.S. Stivala, "Physicochemical Studies of Fractionated Bovine Heparin: Viscosity as a Function of Ionic Strength," Arch. Biochem. Biophys., 119, 510-518 (1967).

FIGURE 5-1



50 mg of heparin was dissolved into 0.5 ml of 1 Molar NaCl solution and loaded onto a 50 cm Sephadex G75 column having a 1.5 cm diameter. The void volume of the column was 26.1 ul by Blue Dextran. The heparin was eluted with 1 Molar NaCl at a flow rate of 160 ul/min at room temperature. Each of the individual fractions collected had a volume of 630 ul. A 10 ul aliquot of each fraction was assayed by the Azure A Assay to determine the heparin concentration in the fraction.

FIGURE 5-2



50 mg of each heparin fraction was dissolved into 0.5 ml of 1 Molar NaCl solution and loaded onto a 50 cm Sephadex G75 column having a 1.5 cm diameter. The void volume of the column was 26.1 ml by Blue dextran. The heparin was eluted with 1 Molar NaCl at a flow rate of 160 ul/min at room temperature. Each of the individual fractions collected had a volume of 630 ul. A 10 ul aliquot of each fraction was assayed by the Azure A Assay to determine the heparin concentration in the fraction.

glycols with molecular weights of 5700 and 3750 (polydispersity = 1.1) were applied as above. The elution of these standards is shown in Figure 5-3. The void volume of the column was measured using Blue Dextran; and its elution is also shown in Figure 5-3.

RESULTS OF THE FRACTIONATION AND STANDARDIZATION.

The void volume of the G75 column was taken as 21.6 ml. The molecular weights of the fractions were found using the equation supplied by Pharmacia for their Sepharose columns[104]

$$\ln Mw = A K_{av} + B \qquad \text{Eq. 5-1}$$

where Mw is the molecular weight of the fraction, A and B are constants determined by suitable molecular weight standards, and K_{av} is defined as

$$K_{av} = (V_{elution} / V_{void}) - 1 \qquad \text{Eq. 5-2}$$

Using the elution volumes of the polyethylene glycols, A = -0.727 and B = 10.291. The five fractions and the original heparin sample thus have the molecular weights as shown in Table 5-1. The precision shown for the molecular weights is much higher than this method is able to give (probably +/- 500), but the extra digits helped to identify the fractions, and so were left.

SENSITIVITY OF THE AZURE A ASSAY.

A standard curve of absorbance at vis 620nm from the Azure A assay vs. concentration (mg/ml) of heparin was made for each molecular weight fraction

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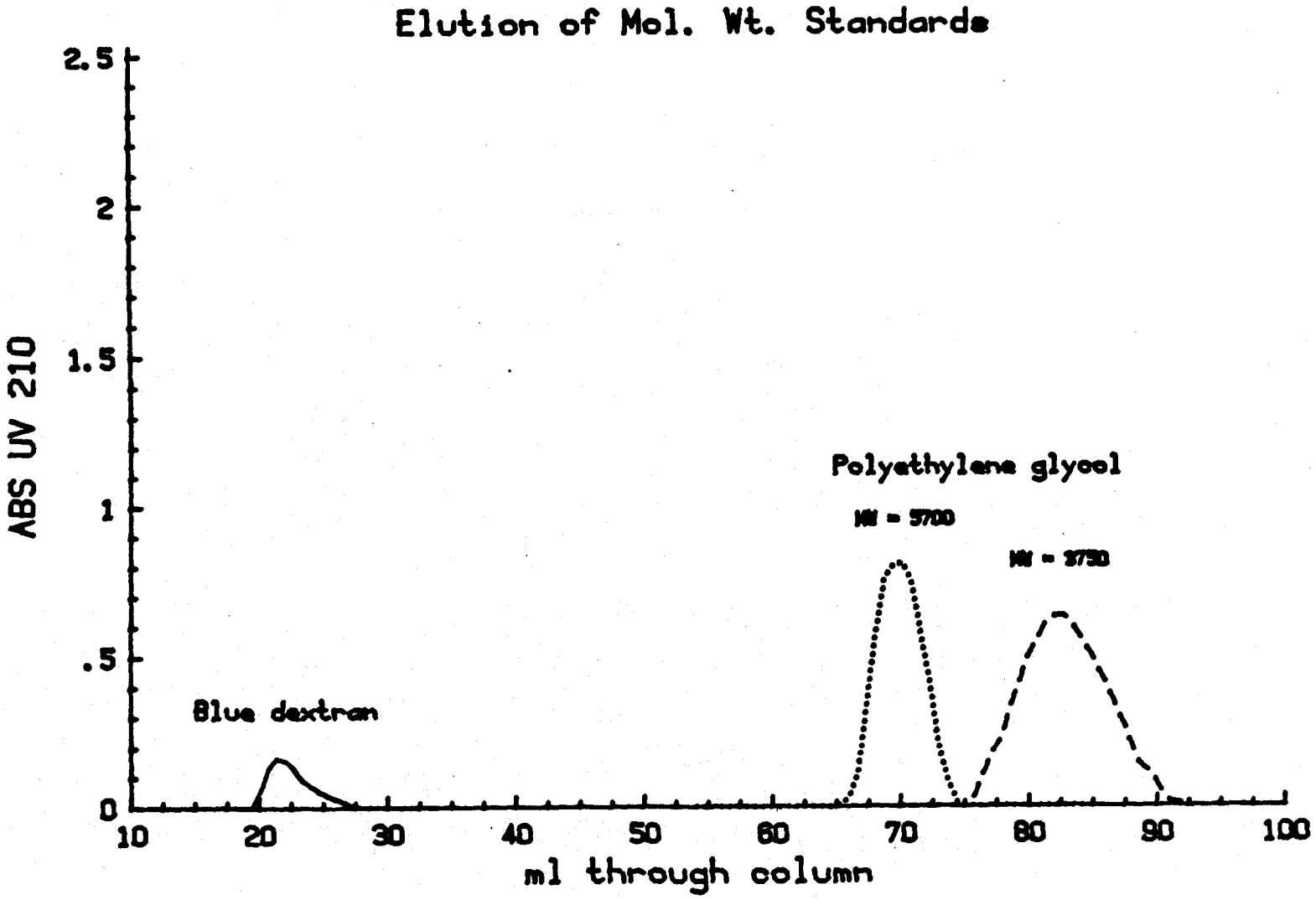
104. Pharmacia Fine Chemicals Co., Affinity Chromatography: Principles and Methods. Pharmacia Fine Chemicals, Uppsala, Sweden.

FIGURE 5-3



500 ul of solution containing 10 mg of Blue dextran, 10 mg of polyethylene glycol (MW 5700) , 10 mg of polyethylene glycol (MW 3750) and 1 Molar NaCl was loaded onto a Sephadex G75 column having a 1.5 cm diameter. The void volume of the column was taken to be 26.1 ml, at the peak of the Blue dextran elution.

Table 5-1. Molecular weights of the heparin fractions

Name of fraction	Peak elution volume	K_{av}	Molecular weight
Original sample	46.341	1.1454	12814
Low MW	57.996	1.6850	8656
Med Low MW	50.841	1.3537	11013
Med MW	41.564	.92426	15049
Med Hi MW	38.404	.77794	16738
High MW	33.060	.53056	20036

The peak elution volumes are taken from Figures 5-1 and 5-2. K_{av} is computed using Equation 5-2. The average molecular weight of the fraction is computed from K_{av} using Equation 5-1.

of heparin and the original sample. These standard curves are shown as Figures 5-4a-f. There is no correlation between the molecular weight of the heparin used to make the standard curve and the slope of the curve. The curves are, in fact, almost completely identical. The mean of the slopes is -0.1768 with a standard deviation of 0.0015. Based on the analysis presented in the theory section; therefore, the Azure A assay is sensitive to the weight of heparin in solution, and not the number of moles.

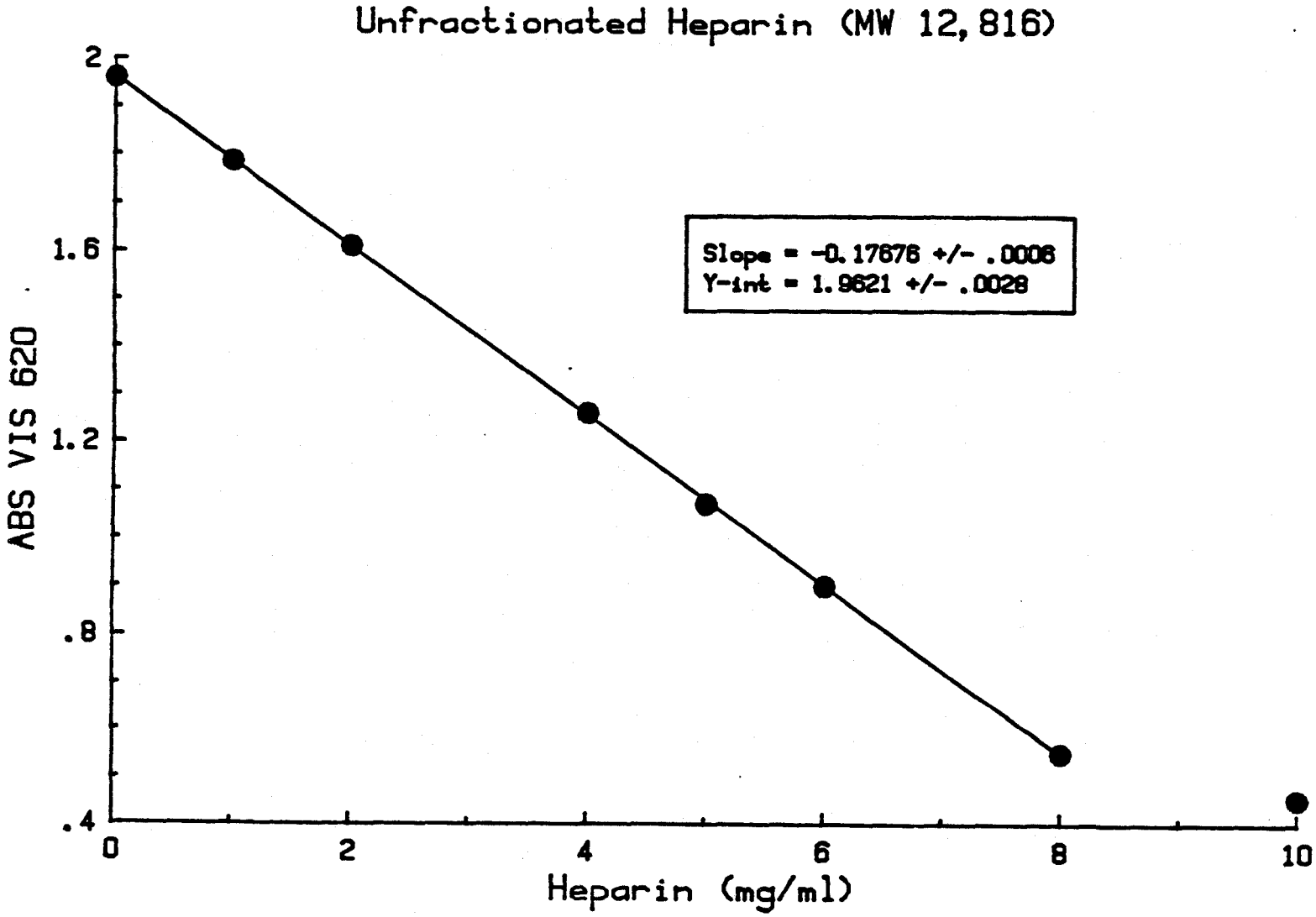
DEGRADATION OF HEPARIN WITH HEPARINASE.

In the following experiment, heparinase (CP) was allowed to degrade each of the fractions and the original sample. The rate of each reaction was followed by the UV 232 assay to determine if the enzyme had an affinity for large or small chains. The endpoint of the assay was used to determine the percentage of cleavable linkages in the heparin sample and in each fraction. A small fraction of each endpoint was left to incubate with the heparinase preparation to test for glycuronidase activity. Finally, the end products of the reaction were run on a fractogel column to determine the final composition in each reacting tube.

PROCEDURE OF THE ASSAY.

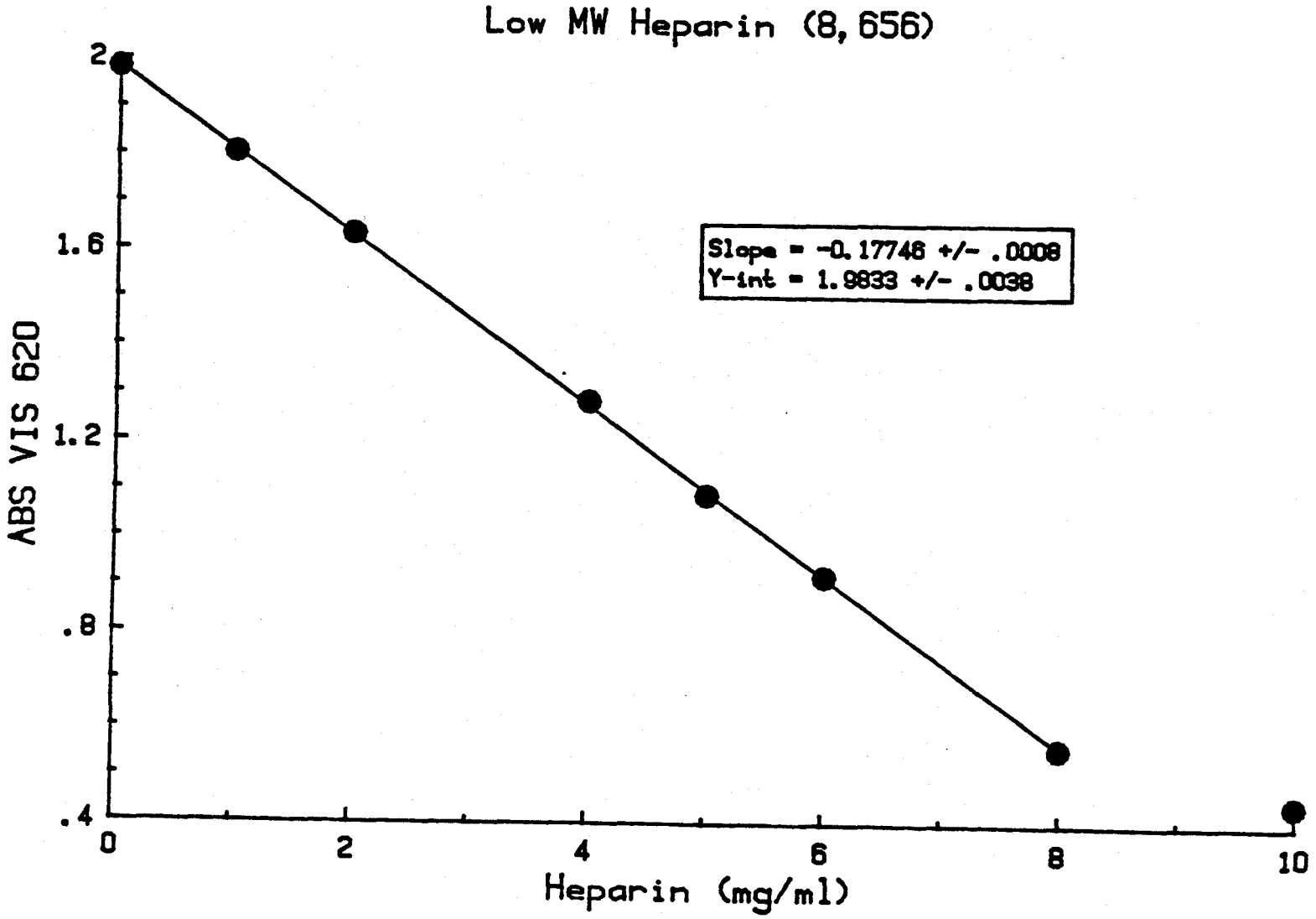
Primary assay tubes were prepared containing 75 ul of heparinase (CP), 250 ul of heparin in OAM, and 425 ul of OAM, so that the total volume was 750 ul. The final heparin concentrations of the assay tubes were 37.5, 25, 12.5, 7.5, 3.0, 2.0, and 1.5 mg/ml. Each primary assay tube was assayed in triplicate by removing a 25 ul aliquot from the primary assay tube and diluting it into 1.25 ml of .03 M HCl and measuring the optical absorbance of

FIGURE 5-4A. AZURE A ASSAY STANDARD CURVE



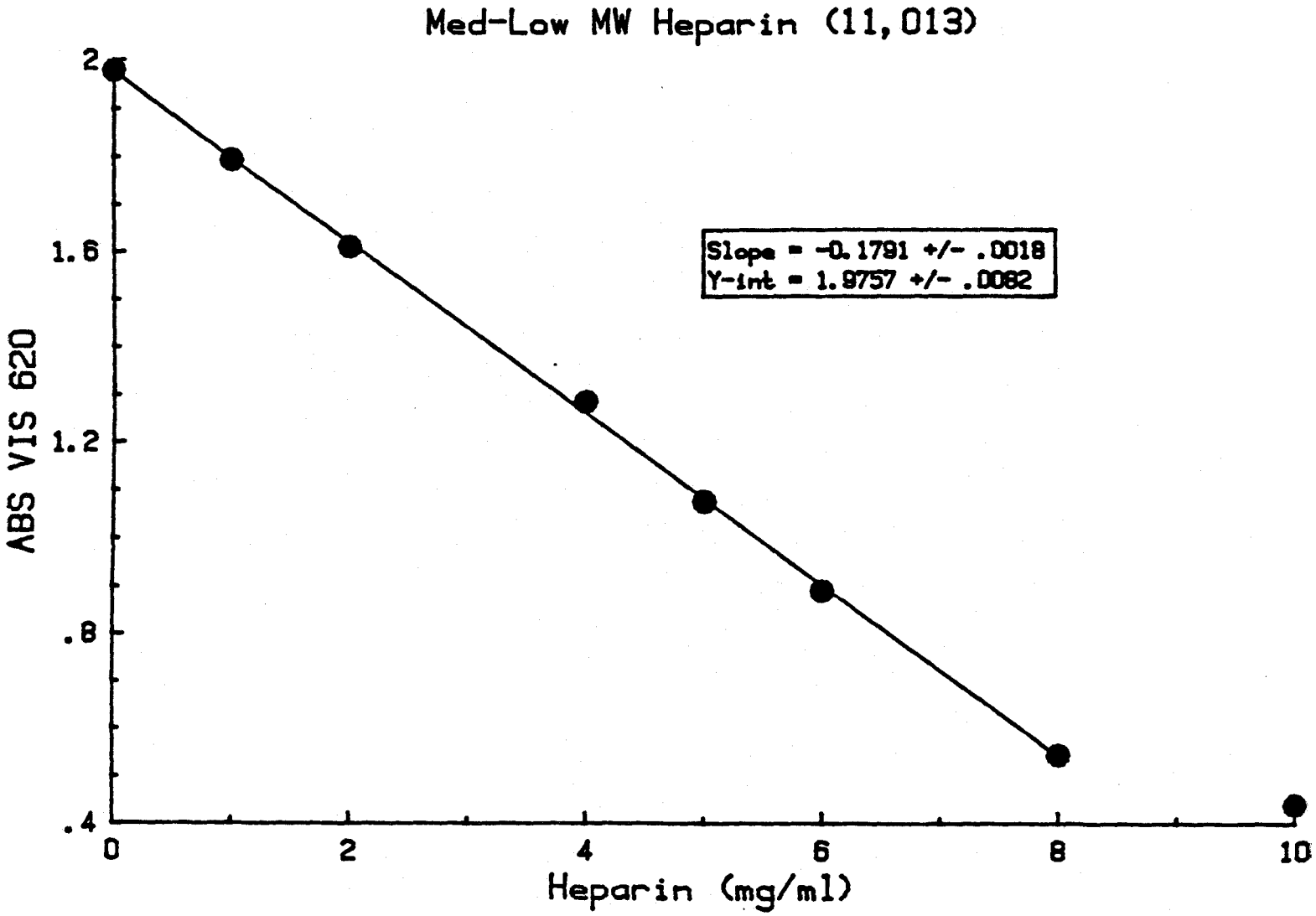
The Standard Curve for the Azure A Assay was prepared by taking a 10 ul aliquot of a solution of heparin in distilled water into 10 ml of a 0.2 g/l solution of Azure A dye. The solutions contained heparin at concentrations of 0,1,2,4,5,6,8 and 10 mg/ml. The absorption of the dye solution at visible 620 nm was measured on a spectrophotometer to quantitate the color change from blue to violet. The response of the assay is linear for heparin solutions with concentrations from 0-8 mg/ml.

FIGURE 5-4B. AZURE A ASSAY STANDARD CURVE



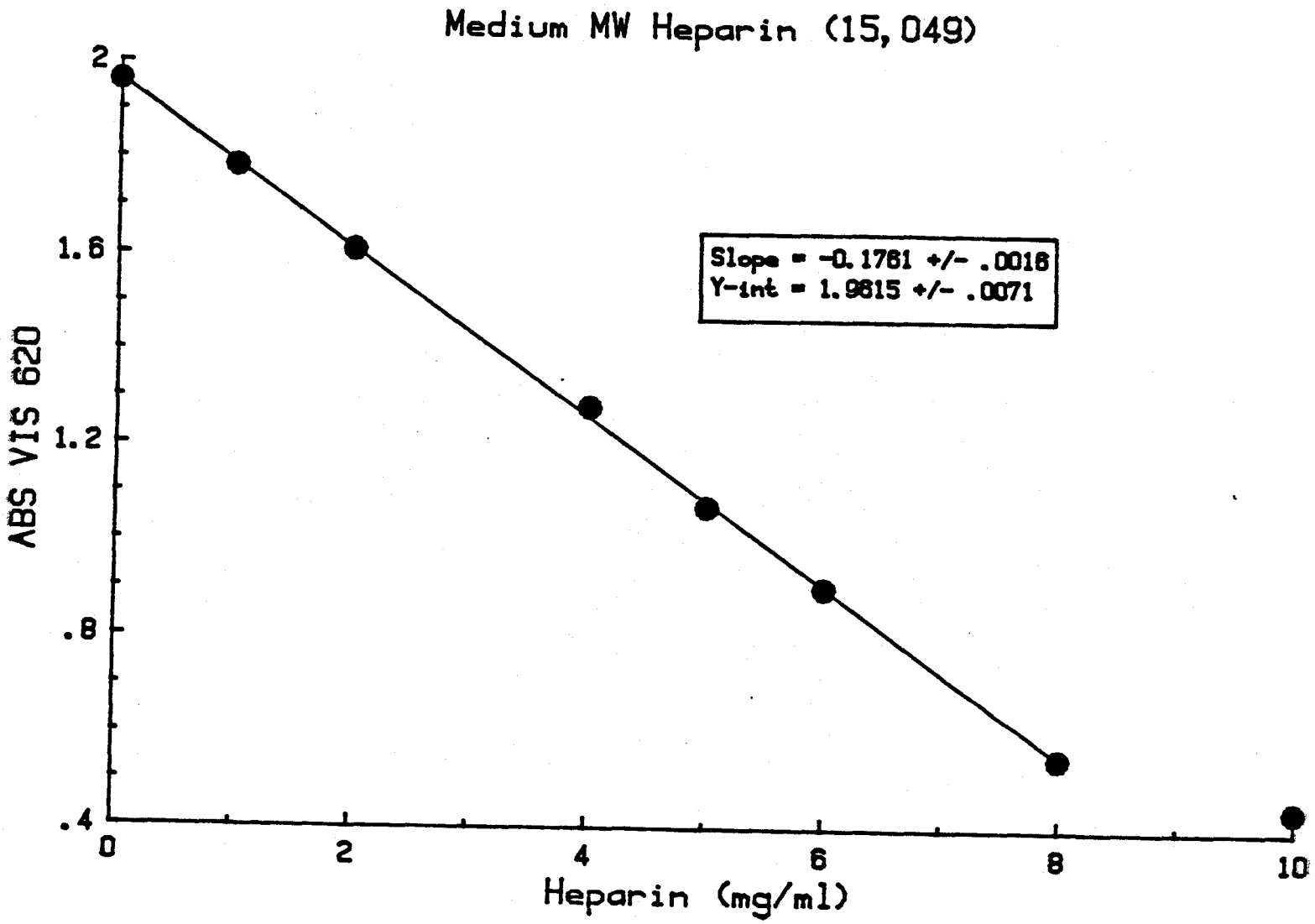
The Standard Curve for the Azure A Assay was prepared by taking a 10 ul aliquot of a solution of heparin in distilled water into 10 ml of a 0.2 g/l solution of Azure A dye. The solutions contained heparin at concentrations of 0,1,2,4,5,6,8 and 10 mg/ml. The absorption of the dye solution at visible 620 nm was measured on a spectrophotometer to quantitate the color change from blue to violet. The response of the assay is linear for heparin solutions with concentrations from 0-8 mg/ml.

FIGURE 5-4c. AZURE A ASSAY STANDARD CURVE



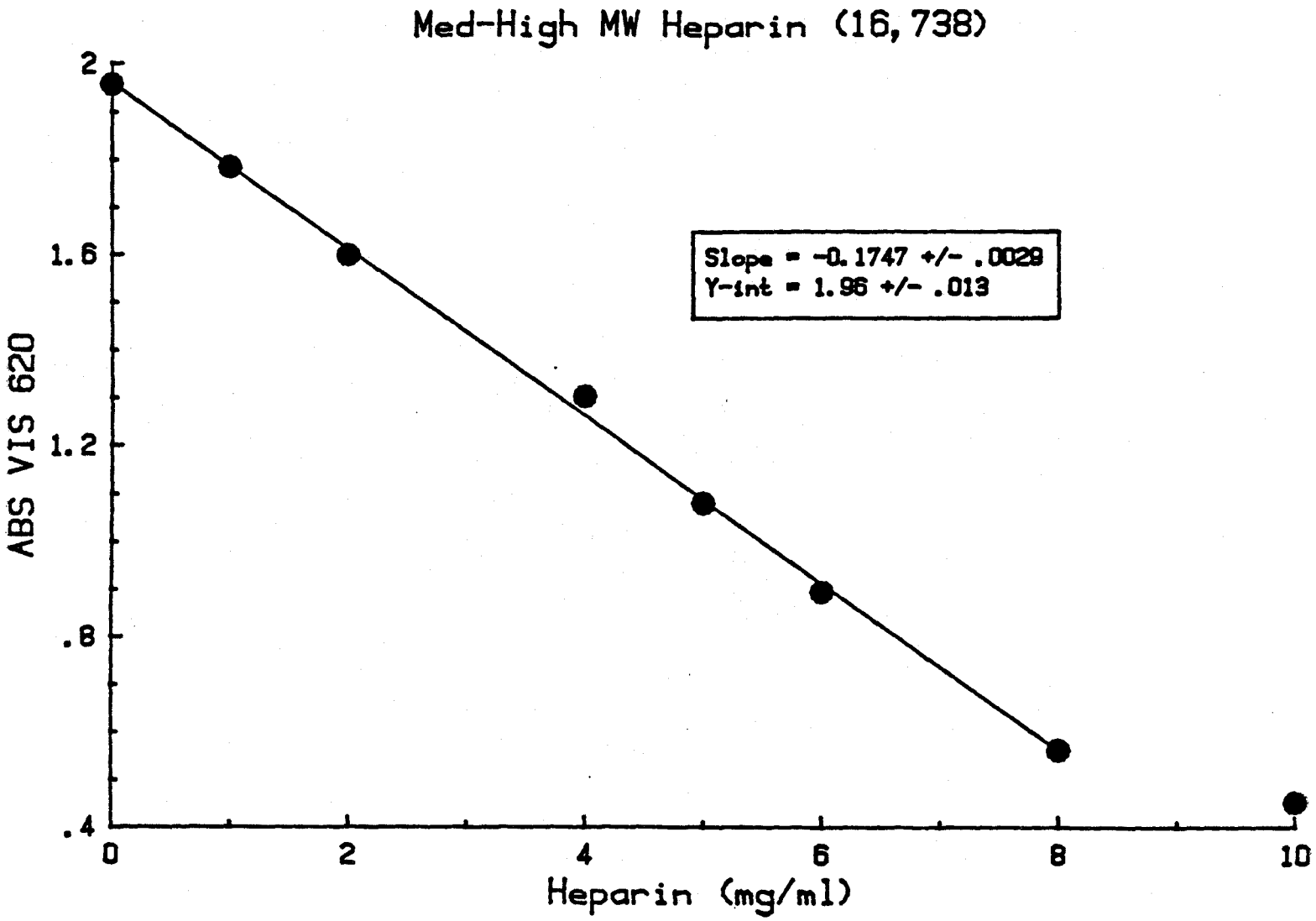
The Standard Curve for the Azure A Assay was prepared by taking a 10 ul aliquot of a solution of heparin in distilled water into 10 ml of a 0.2 g/l solution of Azure A dye. The solutions contained heparin at concentrations of 0,1,2,4,5,6,8 and 10 mg/ml. The absorption of the dye solution at visible 620 nm was measured on a spectrophotometer to quantitate the color change from blue to violet. The response of the assay is linear for heparin solutions with concentrations from 0-8 mg/ml.

FIGURE 5-4D. AZURE A ASSAY STANDARD CURVE



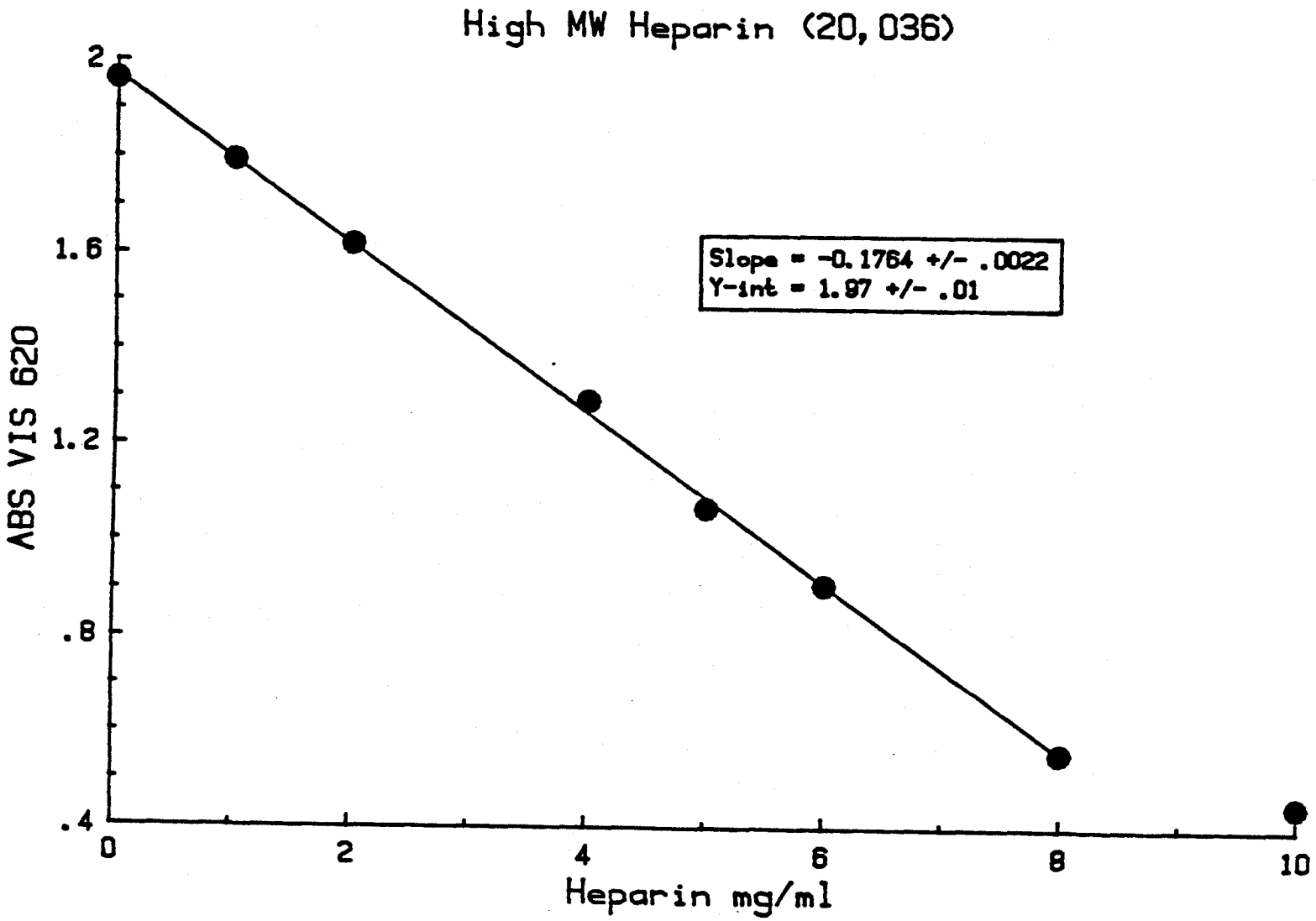
The Standard Curve for the Azure A Assay was prepared by taking a 10 ul aliquot of a solution of heparin in distilled water into 10 ml of a 0.2 g/l solution of Azure A dye. The solutions contained heparin at concentrations of 0,1,2,4,5,6,8 and 10 mg/ml. The absorption of the dye solution at visible 620 nm was measured on a spectrophotometer to quantitate the color change from blue to violet. The response of the assay is linear for heparin solutions with concentrations from 0-8 mg/ml.

FIGURE 5-4E. AZURE A ASSAY STANDARD CURVE



The Standard Curve for the Azure A Assay was prepared by taking a 10 ul aliquot of a solution of heparin in distilled water into 10 ml of a 0.2 g/l solution of Azure A dye. The solutions contained heparin at concentrations of 0,1,2,4,5,6,8 and 10 mg/ml. The absorption of the dye solution at visible 620 nm was measured on a spectrophotometer to quantitate the color change from blue to violet. The response of the assay is linear for heparin solutions with concentrations from 0-8 mg/ml.

FIGURE 5-4F. AZURE A ASSAY STANDARD CURVE



The Standard Curve for the Azure A Assay was prepared by taking a 10 ul aliquot of a solution of heparin in distilled water into 10 ml of a 0.2 g/l solution of Azure A dye. The solutions contained heparin at concentrations of 0,1,2,4,5,6,8 and 10 mg/ml. The absorption of the dye solution at visible 620 nm was measured on a spectrophotometer to quantitate the color change from blue to violet. The response of the assay is linear for heparin solutions with concentrations from 0-8 mg/ml.

the dilution at UV 232nm. The primary assay tubes were assayed at times of reaction of 0, 10, 20, 30, 50, 180, and 410 minutes. After 410 minutes, two 25 ul aliquots were removed from the primary assay tube and placed into endpoint assay tubes. The first endpoint assay tube contained 175 ul of a solution of the original heparin sample at 25 mg/ml in OAM. The second endpoint assay tube was empty. The primary assay tube was then immediately frozen with liquid nitrogen and kept frozen at -20°C until defrosted for chromatography. The first (heparin added) endpoint assay tube was assayed immediately and after 1 hour by the Azure A assay to verify that the heparinase was still active. The second endpoint assay tube was assayed after 3 hours by adding 1.25 ml of 0.03M HCl and measuring the OD of the solution at UV 232nm. The second endpoint assay tube was used to determine the activity of glycuronidases in the enzyme preparation. All reactions were carried out in sealed tubes in a water bath at 30°C (+/- .5°C).

RATES OF REACTION AND MICHAELIS CONSTANTS FOR HEPARIN FRACTIONS.

The initial rates of reaction obtained in each assay tube are given in Table 5-2a in ΔOD 232 nm per hour. Using an extinction coefficient of 5.5 x 10^3 OD units per molar per cm, and a one cm cuvette, these rates were converted to units of moles (of product) per liter per second. The converted rates are shown in Table 5-2b. These rates were used to obtain rate constants for the Michaelis-Menten enzyme kinetics equation by using a Lineweaver-Burk double reciprocal plot of the initial rates and substrate concentrations. These constants are shown in Table 5-3. All of the first endpoint assay tubes (for heparinase activity) showed a decrease of at least 0.1 OD units at 620nm from the last kinetic assay point, indicating that the enzyme, though

Table 5-2a. Rates of reaction for soluble enzyme

Rates expressed as OD UV232nm / hour
Standard errors in parentheses

Heparin fraction	Substrate concentration (mg/ml) 37.5	25.0	12.5	7.50	3.00	2.00	1.50
Original Sample (12814)	7.80 (.008)	7.35 (.010)	7.11 (.016)	6.91 (.008)	6.59 (.020)	6.34 (.028)	6.10 (.040)
Low MW (8656)	7.03 (.006)	7.09 (.008)	6.97 (.024)	6.87 (.028)	6.69 (.022)	6.53 (.028)	6.34 (.034)
Med. Low MW (11013)	7.01 (.009)	7.03 (.012)	7.05 (.018)	6.79 (.016)	6.77 (.026)	6.44 (.032)	6.26 (.055)
Med. MW (15049)	7.17 (.016)	6.97 (.010)	6.95 (.008)	6.85 (.016)	6.55 (.010)	6.30 (.016)	5.94 (.046)
Med. High MW (16738)	7.09 (.020)	6.97 (.022)	6.87 (.050)	6.71 (.036)	6.40 (.038)	6.10 (.032)	5.98 (.066)
High MW (20036)	7.19 (.042)	7.15 (.026)	6.73 (.058)	6.91 (.048)	6.28 (.038)	5.94 (.060)	5.54 (.094)

Assay tubes containing 75 ul of cellulose phosphate purified heparinase, 250 ul of heparin in OAM and 425 ul of OAM with the final heparin concentrations shown were prepared. The assay tubes were assayed by the UV 232 nm assay at times of 0, 10, 20, 30, and 50 minutes after the start of the reaction. Linear regression of the optical absorbance at UV 232 nm over time yielded the reaction rates shown.

Table 5-2b. Rates of reaction for soluble enzyme

Rates expressed as $mol_{(product)}$ / l-s $x10^7$:
Standard errors in parentheses

Heparin fraction	Substrate concentration (mg/ml) 37.5	25.0	12.5	7.50	3.00	2.00	1.50
Original Sample (12814)	3.94 (.004)	3.71 (.005)	3.59 (.008)	3.49 (.004)	3.33 (.010)	3.20 (.014)	3.08 (.020)
Low MW (8656)	3.55 (.003)	3.58 (.004)	3.52 (.012)	3.47 (.014)	3.38 (.011)	3.30 (.014)	3.20 (.017)
Med. Low MW (11013)	3.54 (.0045)	3.55 (.006)	3.56 (.009)	3.43 (.008)	3.42 (.013)	3.25 (.016)	3.16 (.028)
Med. MW (15049)	3.62 (.008)	3.52 (.005)	3.51 (.004)	3.46 (.008)	3.31 (.005)	3.18 (.008)	3.00 (.023)
Med. High MW (16738)	3.58 (.010)	3.52 (.011)	3.47 (.025)	3.39 (.018)	3.23 (.019)	3.08 (.016)	3.02 (.033)
High MW (20036)	3.63 (.021)	3.61 (.013)	3.40 (.029)	3.49 (.024)	3.17 (.019)	3.00 (.030)	2.80 (.047)

The reaction rates shown in Table 5-2a were converted to rates with units of moles of product formed per liter per second. The conversions were all done assuming an extinction coefficient for the UV chromophore of 5.5×10^3 OD units per mole per liter per cm.

Table 5-3. Michaelis constants for the soluble enzyme

Substrate	K_m (mol/l) $\times 10^5$	V_m (mol/l-s) $\times 10^7$	correlation coeff.
	Standard errors in parentheses		
Original sample (12814)	2.75 (.37)	3.77 (.06)	.9107
Low MW (8656)	2.00 (.11)	3.58 (.02)	.9875
Med. Low MW (11013)	1.48 (.17)	3.57 (.02)	.9389
Med. MW (15049)	2.23 (.14)	3.60 (.02)	.9807
Med. High MW (16738)	2.23 (.16)	3.56 (.03)	.9763
High MW (20036)	3.45 (.23)	3.64 (.04)	.9773

A Lineweaver-Burk double-reciprical plot of the data in Table 5-2b yields these values for the kinetic constants for the reactions between heparinase and the heparin fractions.

weakened, was still active. This result is consistent with previous measurements of the half-life of heparinase activity in similar buffer (NAM, pH 7.0).[105] Only two of the second endpoint assay tubes showed a decrease in absorbance at UV 232 nm of greater than 1% after 3 hours, indicating negligible glycuronidase activity. Similar preparations of heparin have shown similar amounts of glycuronidase activity in this type of heparinase preparation.[106] The rates of reaction and the absorbance of the endpoints of reactions thus reflect the activity and specificity of heparinase.

ENDPOINTS AND THE PERCENT OF CLEAVABLE ALPHA LINKAGES.

The final UV 232 readings for the 37.5 mg/ml and 25.0 mg/ml assay tubes are presented in Tables 5-4a and 5-4b respectively. These tables also show the calculations of the percent of alpha linkages that are cleaved during the reaction. For example, the original sample has an estimated number average molecular weight of 12814. This corresponds to an average of 20.025 disaccharides with an average molecular weight of 640. There is therefore an average of 19.025 alpha linkages per heparin molecule. At 37.5 mg/ml the molar concentration of heparin is 2.926×10^{-3}. Multiplying the molar concentration of heparin by the volume of the tube (.75 ml) and the average number of alpha linkages per molecule gives the number of moles of alpha linkages per assay tube (4.173×10^{-5} for the original sample). The final reading of the absorbance in the UV 232 nm assay is converted to a molar concentration using

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105. See Appendix D

106. Linhardt, R.J., G.L. Fitzgerald, C.L. Cooney, and R. Langer, "Mode of Action of Heparin Lyase on Heparin," Biochim. Biophys. Acta, 702, 197-203 (1982).

Table 5.4a. Percent of Cleavable sites from 37.5 mg/ml Assay Tubes.

FRACTION	EST. # links per chain	mol/l heparin x 10^3	mol links per tube x10^5	Final UV reading OD units	Final mol links cleaved per tube x10^5	% links cleaved
Original sample (12814)	19.025	2.926	4.175	1.338	1.825	43.71
Low MW (8656)	12.525	4.332	4.069	1.284	1.751	43.02
Med. Low MW (11013)	16.208	3.405	4.139	1.357	1.850	44.70
Med. MW (15049)	22.514	2.492	4.208	1.358	1.852	44.01
Med. High MW (16738)	25.153	2.240	4.226	1.358	1.852	43.82
High MW (20036)	30.306	1.872	4.255	1.348	1.838	43.20
					mean =	43.74
					std. dev. =	0.60

This table shows the final reading of the UV 232 nm assays for the assay tubes that had an initial heparin concentration of 37.5 mg/ml. This table also shows the calculations of the percent of alpha linkages that are cleaved during the reaction. For example, the original sample has an estimated number average molecular weight of 12814. This corresponds to an average of 20.025 disaccharides (assuming an average molecular weight of 640). There is therefore an average of 19.025 alpha linkages per heparin molecule. At 37.5 mg/ml the molar concentration of heparin was 2.926×10^{-3} initially. Multiplying the molar concentration of heparin by the volume of the tube (.75 ml) and the average number of alpha linkages per molecule gives the number of moles of alpha linkages per assay tube (4.173×10^{-5} for the original sample). The final reading of the absorbance in the UV 232 nm assay is converted to a molar concentration using an extintion coefficient of 5.5×10^3 (calculation not shown). This concentration is then corrected for the dilution of the aliquot (.025 ml to 1.25 ml) and the sampling size (.025 ml from .750 ml) and finally multiplied by the original volume of the assay tube (.750 ml) to obtain the number of moles of UV 232 nm chromophore produced (and therefore the number of alpha linkages cleaved) by the enzyme (1.825×10^{-5} moles for the original sample). The percentage of alpha linkages cleaved is calculated as the ratio between the number of alpha linkages cleaved by the enzyme and the number of alpha linkages estimated to have been in the assay tube initially, multiplied by 100.

Table 5.4b. Percent of Cleavable sites from 25.0 mg/ml Assay Tubes.

FRACTION	EST. # links per chain	mol/l heparin x 10^3	mol links per tube $x10^5$	Final UV reading OD units	Final mol links cleaved per tube $x10^5$	% links cleaved
Original sample (12814)	19.025	1.951	2.783	.895	1.220	43.85
Low MW (8656)	12.525	2.888	2.713	.877	1.196	44.08
Med. Low MW (11013)	16.208	2.270	2.759	.891	1.215	44.04
Med. MW (15049)	22.514	1.661	2.805	.912	1.244	44.33
Med. High MW (16738)	25.153	1.494	2.818	.900	1.227	43.55
High MW (20036)	30.306	1.248	2.836	.903	1.231	43.42
					mean =	43.88
					std. dev. =	0.34

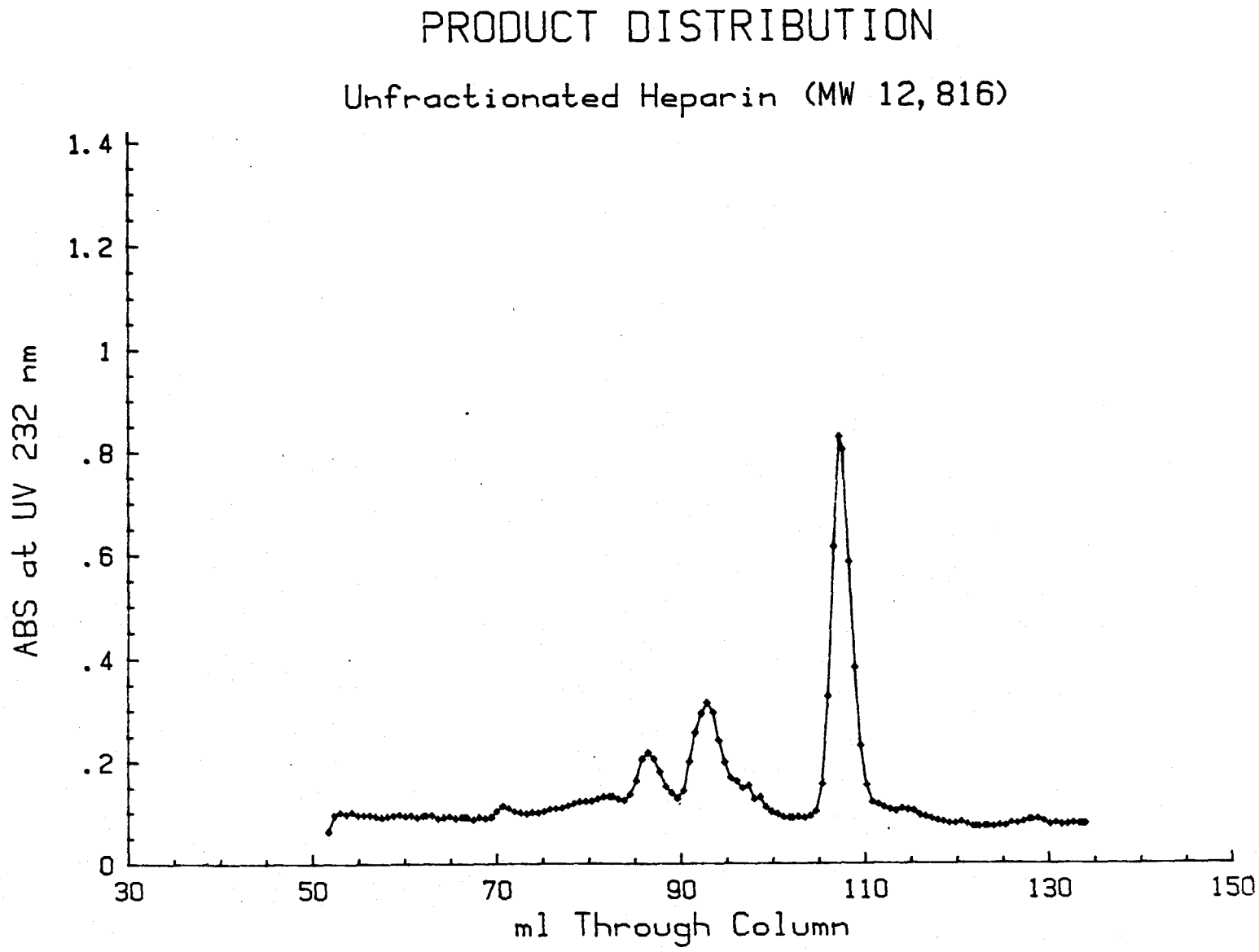
This table shows the final reading of the UV 232 nm assays for the assay tubes that had an initial heparin concentration of 37.5 mg/ml. This table also shows the calculations of the percent of alpha linkages that are cleaved during the reaction. For example, the original sample has an estimated number average molecular weight of 12814. This corresponds to an average of 20.025 disaccharides (assuming an average molecular weight of 640). There is therefore an average of 19.025 alpha linkages per heparin molecule. At 25.0 mg/ml the molar concentration of heparin was 1.9516×10^{-3} initially. Multiplying the molar concentration of heparin by the volume of the tube (.75 ml) and the average number of alpha linkages per molecule gives the number of moles of alpha linkages per assay tube (2.783×10^{-5} for the original sample). The final reading of the absorbance in the UV 232 nm assay is converted to a molar concentration using an extintion coefficient of 5.5×10^3 (calculation not shown). This concentration is then corrected for the dilution of the aliquot (.025 ml to 1.25 ml) and the sampling size (.025 ml from .750 ml) and finally multiplied by the original volume of the assay tube (.750 ml) to obtain the number of moles of UV 232 nm chromophore produced (and therefore the number of alpha linkages cleaved) by the enzyme (1.220×10^{-5} moles for the original sample). The percentage of alpha linkages cleaved is calculated as the ratio between the number of alpha linkages cleaved by the enzyme and the number of alpha linkages estimated to have been in the assay tube initially, multiplied by 100.

the same extinction coefficient as above (calculation not shown). This concentration is then corrected for the dilution of the aliquot (.025 ml to 1.25 ml) and the sampling size (.025 ml from .750 ml) and finally multiplied by the original volume of the assay tube (.750 ml) to obtain the number of moles of UV 232 nm chromophore produced (and therefore the number of alpha linkages cleaved) by the enzyme (1.825×10^{-5} moles for the original sample). The percentage of alpha linkages cleaved is calculated as the ratio between the number of alpha linkages cleaved by the enzyme and the number of alpha linkages estimated to have been in the assay tube initially, multiplied by 100. This ratio is extremely consistent for all the heparin fractions tested at about 43.8% with a standard deviation of no more than 0.6%.

EXPERIMENTALLY DETERMINED DISTRIBUTION OF PRODUCTS.

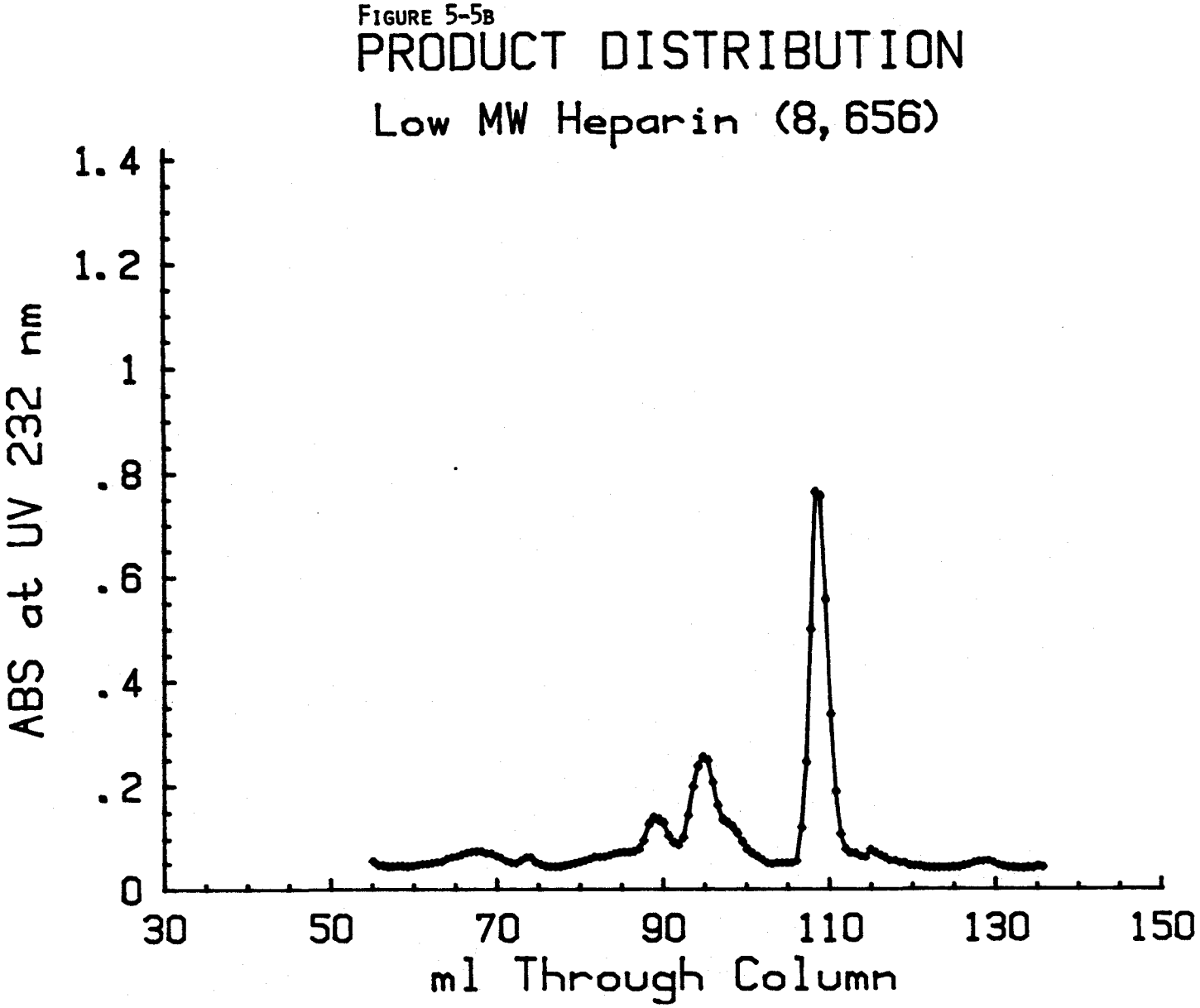
The final product mixtures were defrosted and a small amount (~2 mg of product, based on the initial concentration of heparin in the solution) of the final products were loaded onto the fractogel column and eluted in 1 M NaCl. The elution profile for each fraction of heparin is presented in Figures 5-5, a-f. As the elution of the products at such low flow rates took as much as 24 hours, the baseline for the elution was taken as a line defined by linear regression of all points before the void and between 120 and 130 ml. All of the peaks were integrated by multiplying the difference between the optical density of the tube and the baseline by the volume of the tube and summing the products under each peak. The molar percentages of disaccharide, tetrasaccharide, hexasaccharide, and oligosaccharide products were then calculated. These percentages are given in Tables 5-5a-f. It should be noted that these percentages do not measure the total amount of disaccharide, tetra-

FIGURE 5-5A



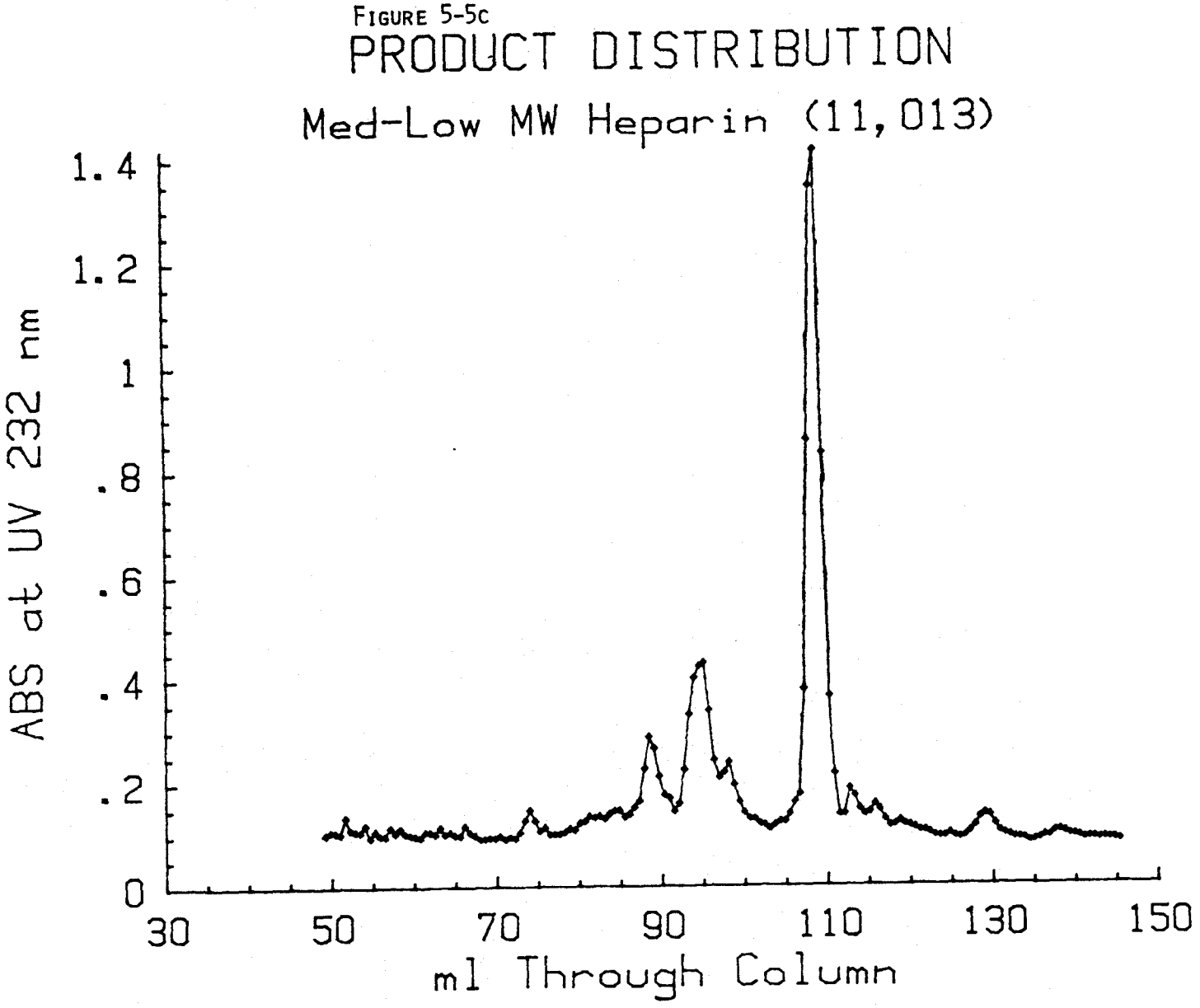
A small (<200 ul) sample of the final product mixture of the heparin-heparinase reaction containing approximately 2 mg of final products was loaded onto a 1.5 cm diameter fractogel column. The column was 1.5 m long and had a void volume of 660 ml by Blue dextran. The products were eluted in 1 Molar NaCl at a flow rate of approximately 100 ul/min. The optical absorption of the eluant was measured at UV 232 nm using 1 Molar NaCl as a blank.

FIGURE 5-5B



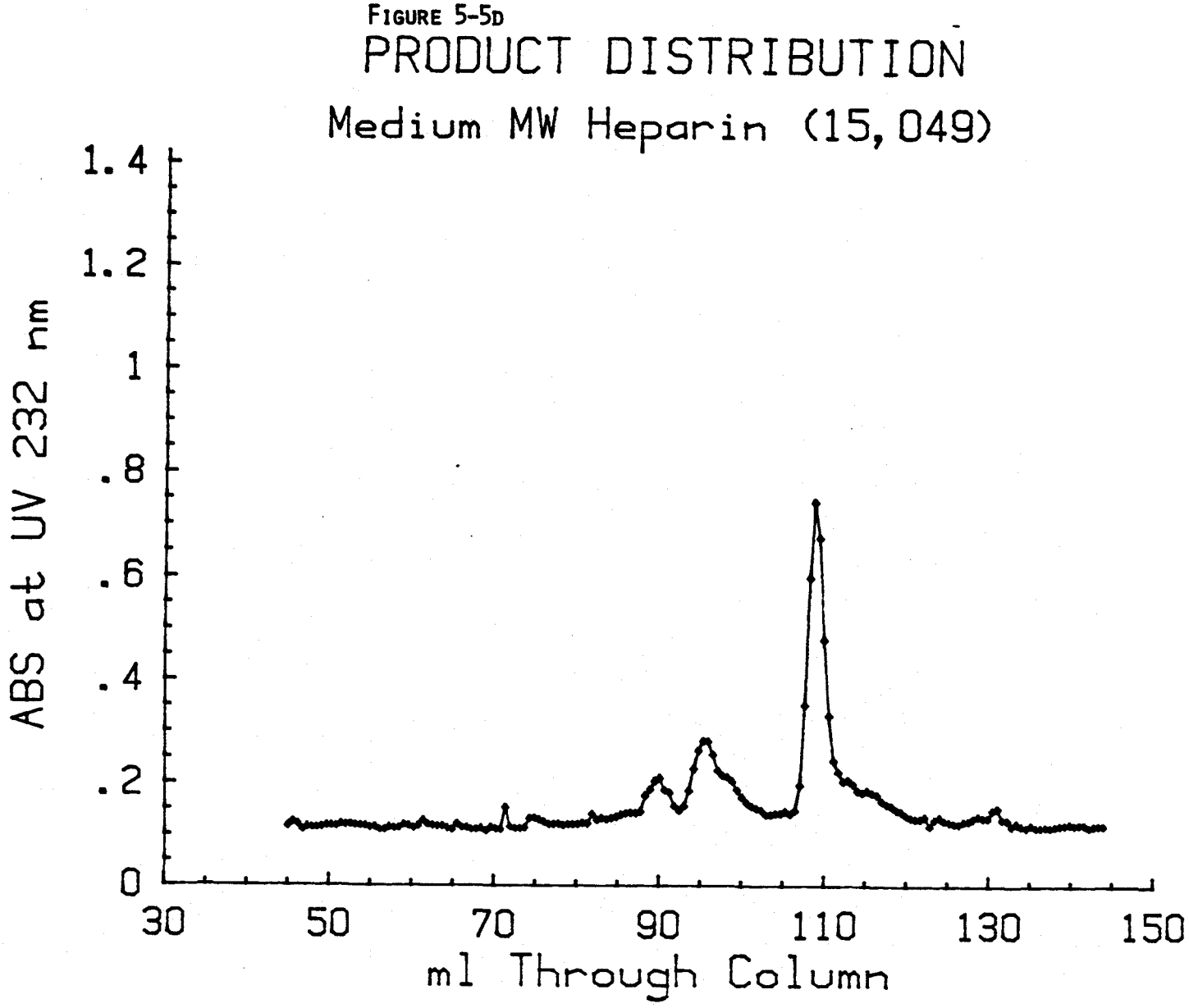
A small (<200 ul) sample of the final product mixture of the heparin-heparinase reaction containing approximately 2 mg of final products was loaded onto a 1.5 cm diameter fractogel column. The column was 1.5 m long and had a void volume of 660 ml by Blue dextran. The products were eluted in 1 Molar NaCl at a flow rate of approximately 100 ul/min. The optical absorption of the eluant was measured at UV 232 nm using 1 Molar NaCl as a blank.

FIGURE 5-5c



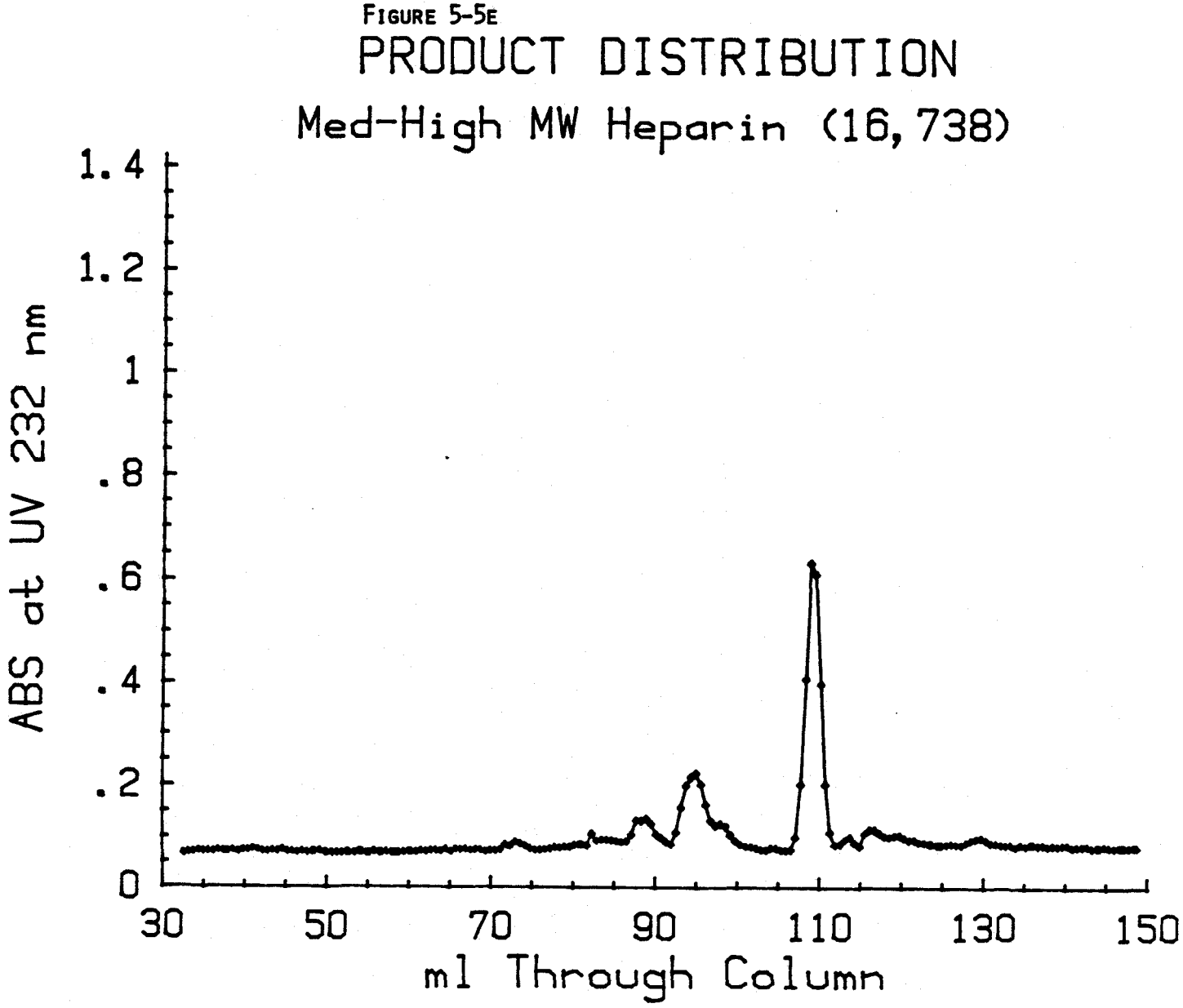
A small (<200 ul) sample of the final product mixture of the heparin-heparinase reaction containing approximately 2 mg of final products was loaded onto a 1.5 cm diameter fractogel column. The column was 1.5 m long and had a void volume of 660 ml by Blue dextran. The products were eluted in 1 Molar NaCl at a flow rate of approximately 100 ul/min. The optical absorption of the eluant was measured at UV 232 nm using 1 Molar NaCl as a blank.

FIGURE 5-5D



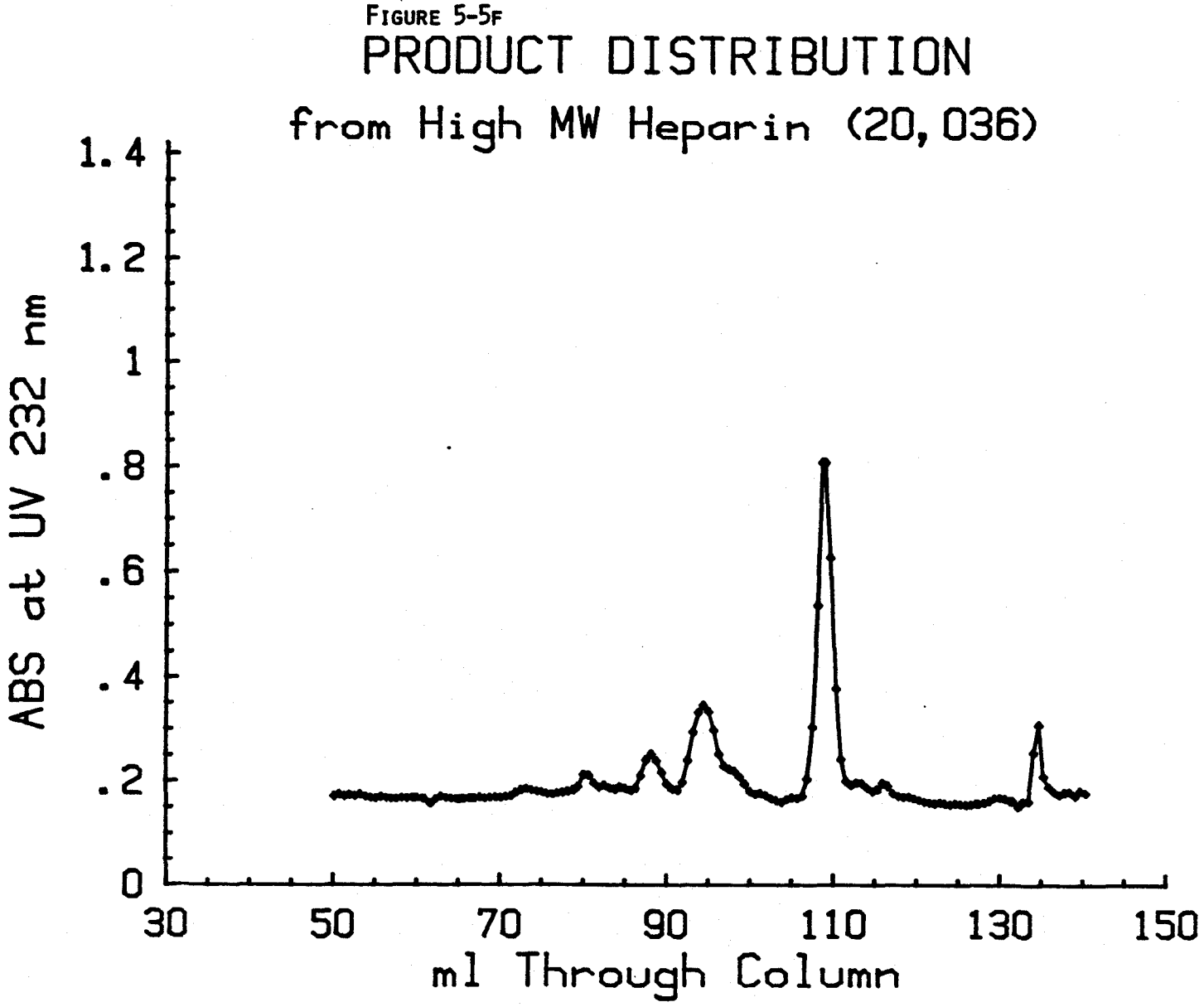
A small (<200 ul) sample of the final product mixture of the heparin-heparinase reaction containing approximately 2 mg of final products was loaded onto a 1.5 cm diameter fractogel column. The column was 1.5 m long and had a void volume of 660 ml by Blue dextran. The products were eluted in 1 Molar NaCl at a flow rate of approximately 100 ul/min. The optical absorption of the eluant was measured at UV 232 nm using 1 Molar NaCl as a blank.

FIGURE 5-5E



A small (<200 ul) sample of the final product mixture of the heparin-heparinase reaction containing approximately 2 mg of final products was loaded onto a 1.5 cm diameter fractogel column. The column was 1.5 m long and had a void volume of 660 ml by Blue dextran. The products were eluted in 1 Molar NaCl at a flow rate of approximately 100 ul/min. The optical absorption of the eluant was measured at UV 232 nm using 1 Molar NaCl as a blank.

FIGURE 5-5F



A small (<200 ul) sample of the final product mixture of the heparin-heparinase reaction containing approximately 2 mg of final products was loaded onto a 1.5 cm diameter fractogel column. The column was 1.5 m long and had a void volume of 660 ml by Blue dextran. The products were eluted in 1 Molar NaCl at a flow rate of approximately 100 ul/min. The optical absorption of the eluant was measured at UV 232 nm using 1 Molar NaCl as a blank.

Table 5-5a. Measured Product Distribution: Unfractionated Heparin

Product	Area (OD ml)	% of total area
Disaccharide	3.918	46.77
Tetrasaccharide	2.113	25.22
Hexasaccharide	1.173	14.00
Oligosaccharide	1.173	14.00
Total	8.377	99.99

The area under each peak in Figure 5-5a was found by multiplying the difference between the optical density of the tube and the baseline by the volume of the tube and summing the products under each peak. The areas for each peak are listed, as is the total area of the peaks. Oligosaccharide refers to any polysaccharide larger than a hexasaccharide. The percentage of each product relative to the total product can then be computed.

Table 5-5b. Measured Product Distribution: Low MW Heparin

Product	Area (OD ml)	% of total area
Disaccharide	3.821	48.74
Tetrasaccharide	1.975	25.19
Hexasaccharide	1.121	14.30
Oligosaccharide	.923	11.77
Total	7.840	100.00

The area under each peak in Figure 5-5b was found by multiplying the difference between the optical density of the tube and the baseline by the volume of the tube and summing the products under each peak. The areas for each peak are listed, as is the total area of the peaks. Oligosaccharide refers to any polysaccharide larger than a hexasaccharide. The percentage of each product relative to the total product can then be computed.

Table 5-5c. Measured Product Distribution: Med Low MW Heparin

Product	Area (OD ml)	% of total area
Disaccharide	6.301	46.39
Tetrasaccharide	3.467	25.52
Hexasaccharide	1.866	13.74
Oligosaccharide	1.949	14.35
Total	13.583	100.00

The area under each peak in Figure 5-5c was found by multiplying the difference between the optical density of the tube and the baseline by the volume of the tube and summing the products under each peak. The areas for each peak are listed, as is the total area of the peaks. Oligosaccharide refers to any polysaccharide larger than a hexasaccharide. The percentage of each product relative to the total product can then be computed.

Table 5-5d. Measured Product Distribution: Med MW Heparin

Product	Area (OD ml)	% of total area
Disaccharide	3.591	47.78
Tetrasaccharide	1.908	25.38
Hexasaccharide	1.051	13.98
Oligosaccharide	.966	12.86
Total	7.516	100.00

The area under each peak in Figure 5-5d was found by multiplying the difference between the optical density of the tube and the baseline by the volume of the tube and summing the products under each peak. The areas for each peak are listed, as is the total area of the peaks. Oligosaccharide refers to any polysaccharide larger than a hexasaccharide. The percentage of each product relative to the total product can then be computed.

Table 5-5e. Measured Product Distribution: Med High MW Heparin

Product	Area (OD ml)	% of total area
Disaccharide	2.730	45.99
Tetrasaccharide	1.552	26.14
Hexasaccharide	.765	12.88
Oligosaccharide	.890	14.99
Total	5.937	100.00

The area under each peak in Figure 5-5e was found by multiplying the difference between the optical density of the tube and the baseline by the volume of the tube and summing the products under each peak. The areas for each peak are listed, as is the total area of the peaks. Oligosaccharide refers to any polysaccharide larger than a hexasaccharide. The percentage of each product relative to the total product can then be computed.

Table 5-5f. Measured Product Distribution: High MW Heparin

Product	Area (OD ml)	% of total area
Disaccharide	3.196	45.46
Tetrasaccharide	1.755	24.97
Hexasaccharide	.989	14.07
Oligosaccharide	1.090	15.50
Total	7.030	100.00

The area under each peak in Figure 5-5f was found by multiplying the difference between the optical density of the tube and the baseline by the volume of the tube and summing the products under each peak. The areas for each peak are listed, as is the total area of the peaks. Oligosaccharide refers to any polysaccharide larger than a hexasaccharide. The percentage of each product relative to the total product can then be computed.

saccharide, or hexasaccharide in the final mixture, but only the fraction of these products which contain the UV 232 nm chromophore. The original non-reducing end of each heparin molecule is not reacted to form this chromophore, and thus the products which contain the original non-reducing ends of the heparin molecules are invisible in this assay. Still, enough data has been collected to perform simulations of the degradation of heparin by heparinase and compare the results of those simulations with measured data. The simulations' fit to the experimental data will be used as the criterion to judge the fitness of the assumptions made in the simulation.

6. DISCUSSION

The product distribution obtained from the degradation of each fraction of heparin and the original sample will be compared with the product distribution predicted by a random, independent distribution of cleavable alpha linkages. In order to make these predictions, the model requires two inputs: the initial molecular weight distribution of the heparin chains, and the percent of the alpha linkages that are heparinase-cleavable. The initial molecular weight distributions for each fraction and the original sample will be inferred by their elution profiles from the G75 column. The percent of the alpha linkages that are heparinase cleavable will be taken from the final absorbance of the product mixture at UV 232nm (see Table 5-4a). The sensitivity of the final product distribution to small amounts of order in the initial distribution of heparinase-cleavable sites will be calculated by repeating the calculations to predict the final distribution of cleavable sites using the diad analysis, triad analysis, and template insertion methods to construct the initial theoretical heparin chains. By comparing the final product distributions obtained experimentally with the distributions predicted by the various models, the amounts of non-randomness or dependence present in the distribution of heparinase-cleavable alpha linkages in heparin can be inferred.

INITIAL MOLECULAR WEIGHT DISTRIBUTION OF HEPARIN FRACTIONS.

The initial molecular weight distributions of the heparin fractions and the original heparin sample can be found from their elution profiles on the G75 column. First, elution profiles for each of the heparin fractions and the original heparin sample were obtained (see Figs. 5-1 and 5-2). The axes of the elution profiles were transformed to obtain molecular weight distributions

by the following method. The x axis of each elution profile was first transformed according to Eq. 5-1, which converts the elution volume to molecular weight. The y axis was first transformed by dividing the concentration at each point by the molecular weight. This gave a plot of molecular weight vs. relative number of moles at that molecular weight. (These plots are not shown.) These plots were then again transformed. The x axis was divided by 640, the average molecular weight of a disaccharide[107], to yield an x axis of "Number of disaccharides per chain". The y axis of each plot was scaled up until the total number of disaccharides in the ensemble of chains was approximately 35,000. This y axis shows the number of chains of each molecular weight in an ensemble of chains containing about 35,000 disaccharides. These plots are shown in Figures 6-1a-f.

The molecular weight distributions given by these plots were approximated by the distributions in Table 6-1. This table gives the number of chains of each length used to model the molecular weight distribution of each fraction of heparin and the original sample. The information in Table 6-1 is also shown by the histogram plots that overlay each of the Figures 6-1a-f.

RELATIVE AFFINITIES BETWEEN HEPARINASE AND VARIOUS MOLECULAR WEIGHT HEPARINS.

Table 5-3 shows the K_m for the reaction between heparinase and each of the five fractions and the original sample of heparin. These K_m's have a mean of 2.05×10^{-5} moles/liter (with a standard deviation of $.39 \times 10^{-5}$ moles/liter). There is no correlation between the molecular weight of the heparin and the K_m for the reaction between heparinase and heparin in the

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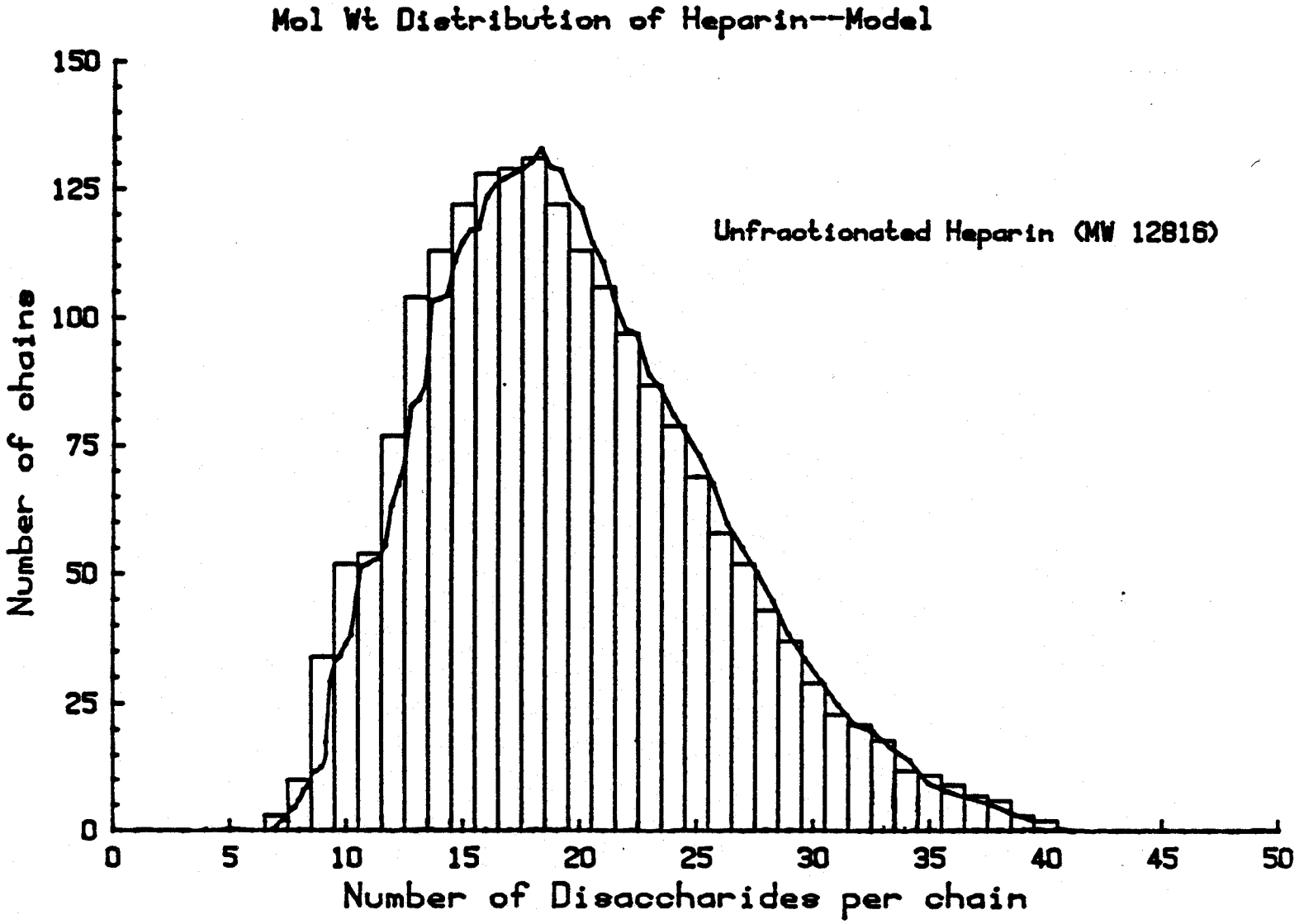
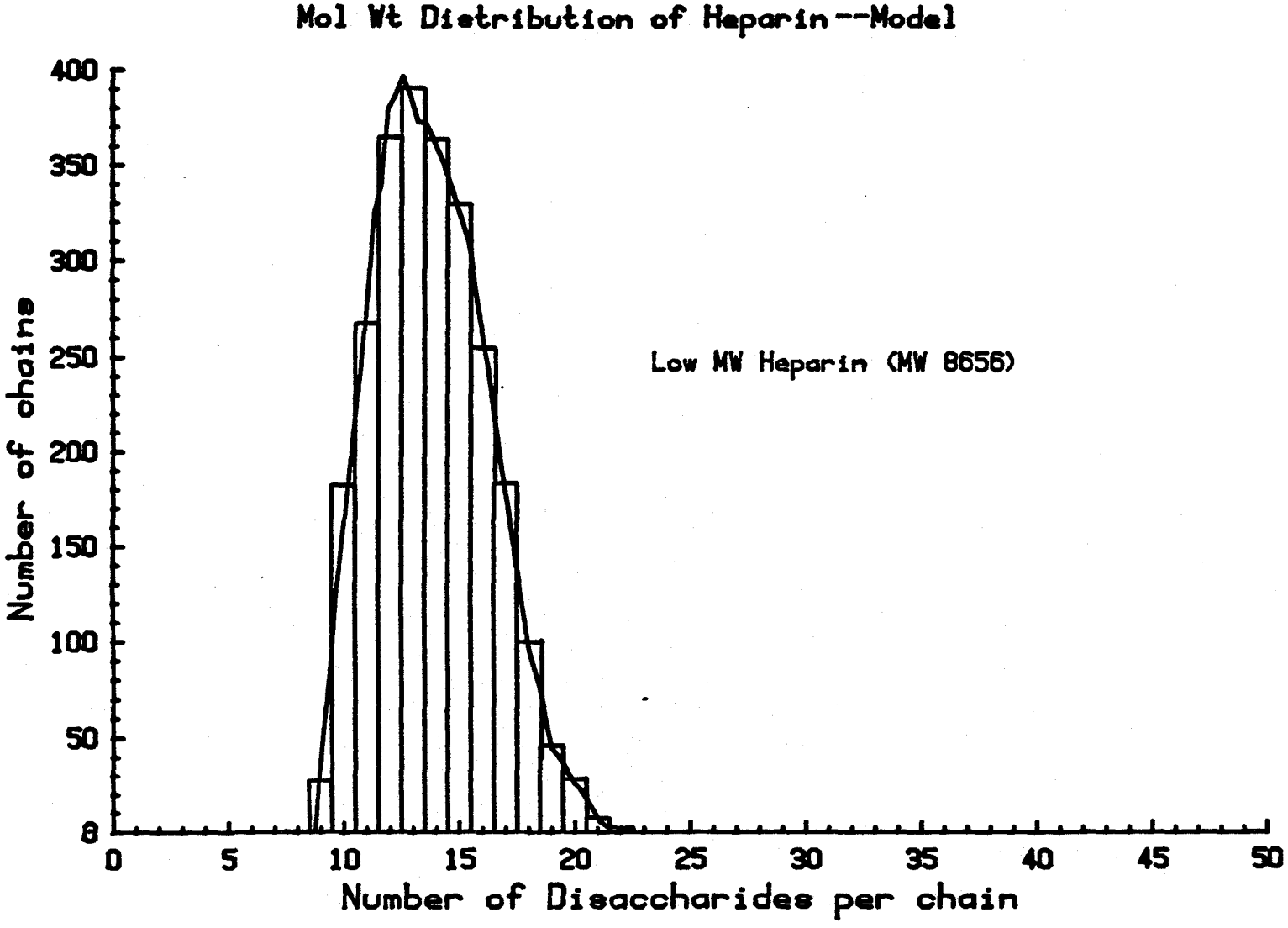
107. Rosenberg, R.D., G. Armand, and L. Lam, "Structure-Function Relationships of Heparin Species," Biochemistry, 75, 3065-3069 (1982).

FIGURE 6-1A



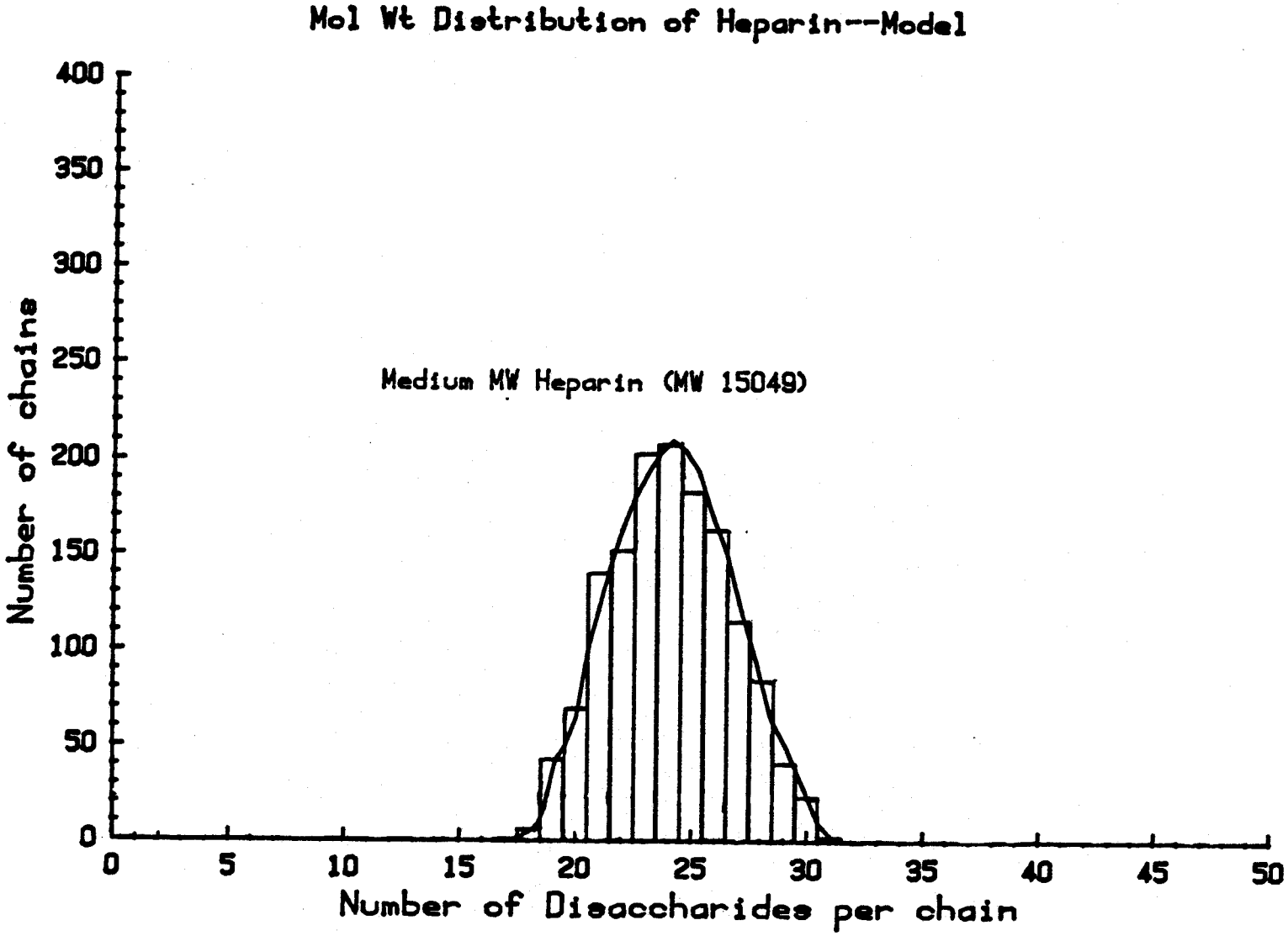
The elution profile for unfractionated heparin presented in Figure 5-1 was converted using Equation 5-1 to show the estimated frequency of each chain length of heparin in unfractionated heparin. The curve shows the relative frequency of each chain length based on the elution pattern of unfractionated heparin. The histogram represents the initial molecular weight distribution of heparin used in the computer simulation of the degradation of unfractionated heparin by heparinase.

FIGURE 6-1B



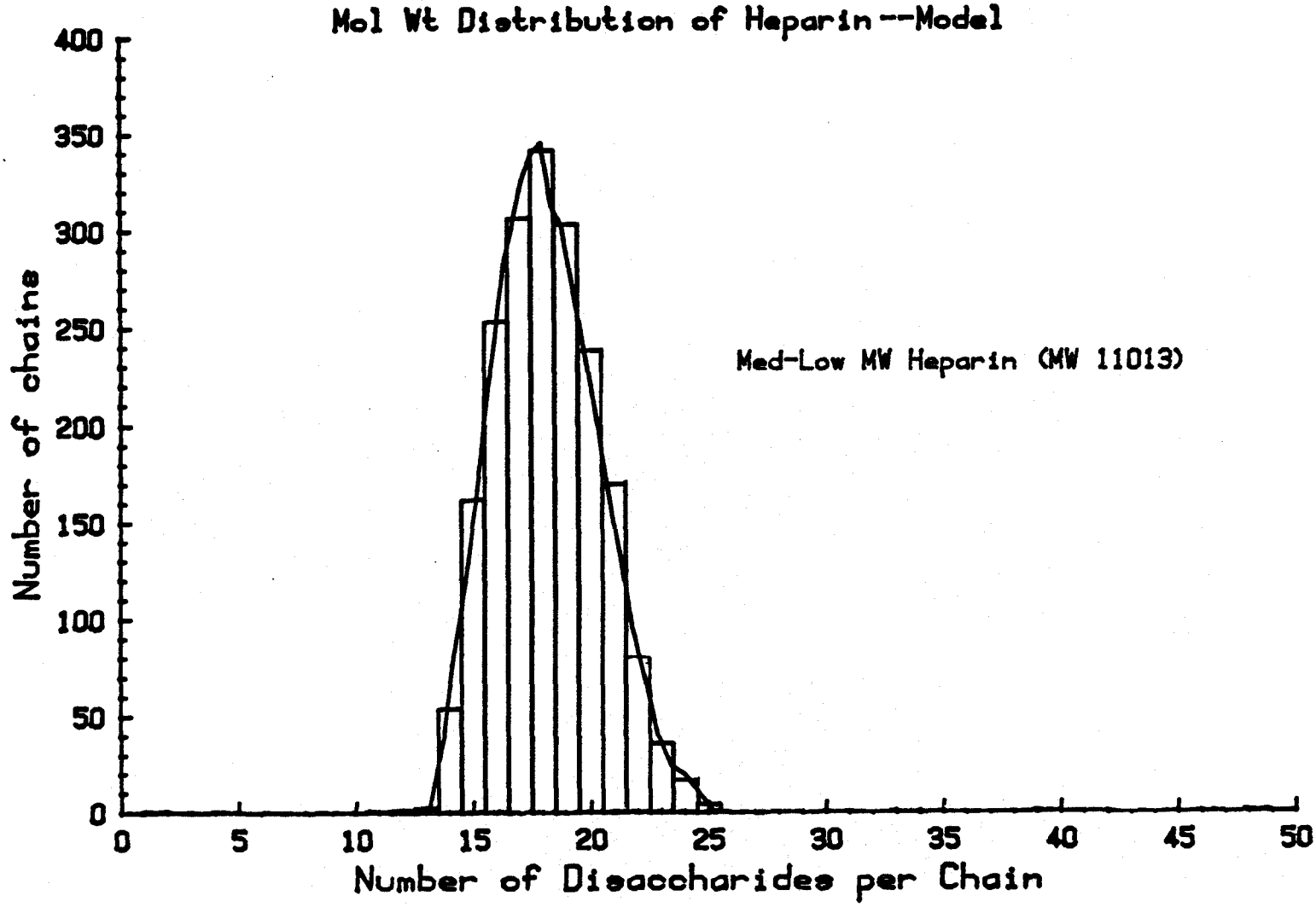
The elution profile for low molecular weight heparin presented in Figure 5-2 was converted using Equation 5-1 to show the estimated frequency of each chain length of heparin in low molecular weight heparin. The curve shows the relative frequency of each chain length based on the elution pattern of low molecular weight heparin. The histogram represents the initial molecular weight distribution of heparin used in the computer simulation of the degradation of low molecular weight heparin by heparinase.

FIGURE 6-1c



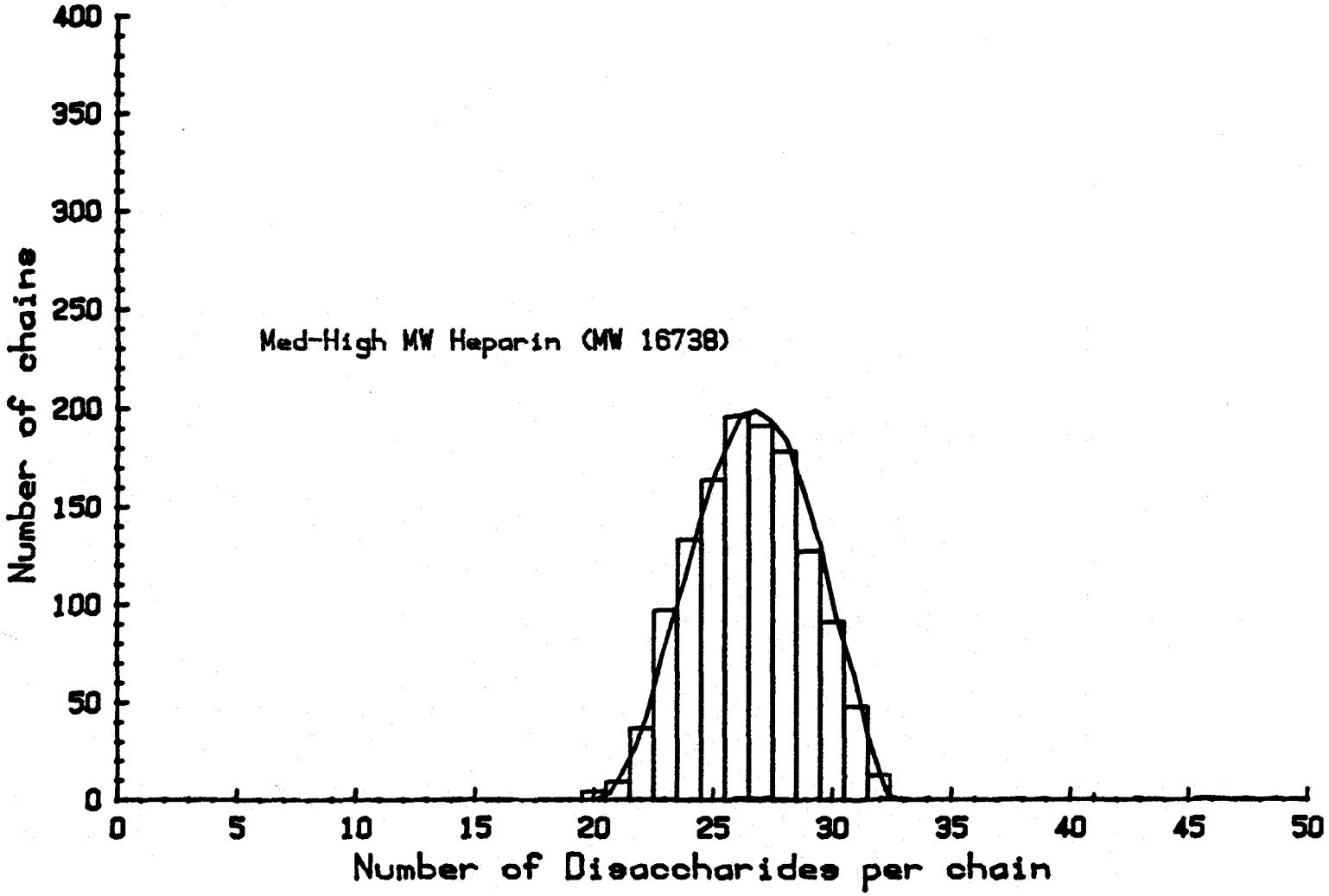
The elution profile for medium molecular weight heparin presented in Figure 5-2 was converted using Equation 5-1 to show the estimated frequency of each chain length of heparin in medium molecular weight heparin. The curve shows the relative frequency of each chain length based on the elution pattern of medium molecular weight heparin. The histogram represents the initial molecular weight distribution of heparin used in the computer simulation of the degradation of medium molecular weight heparin by heparinase.

FIGURE 6-1D



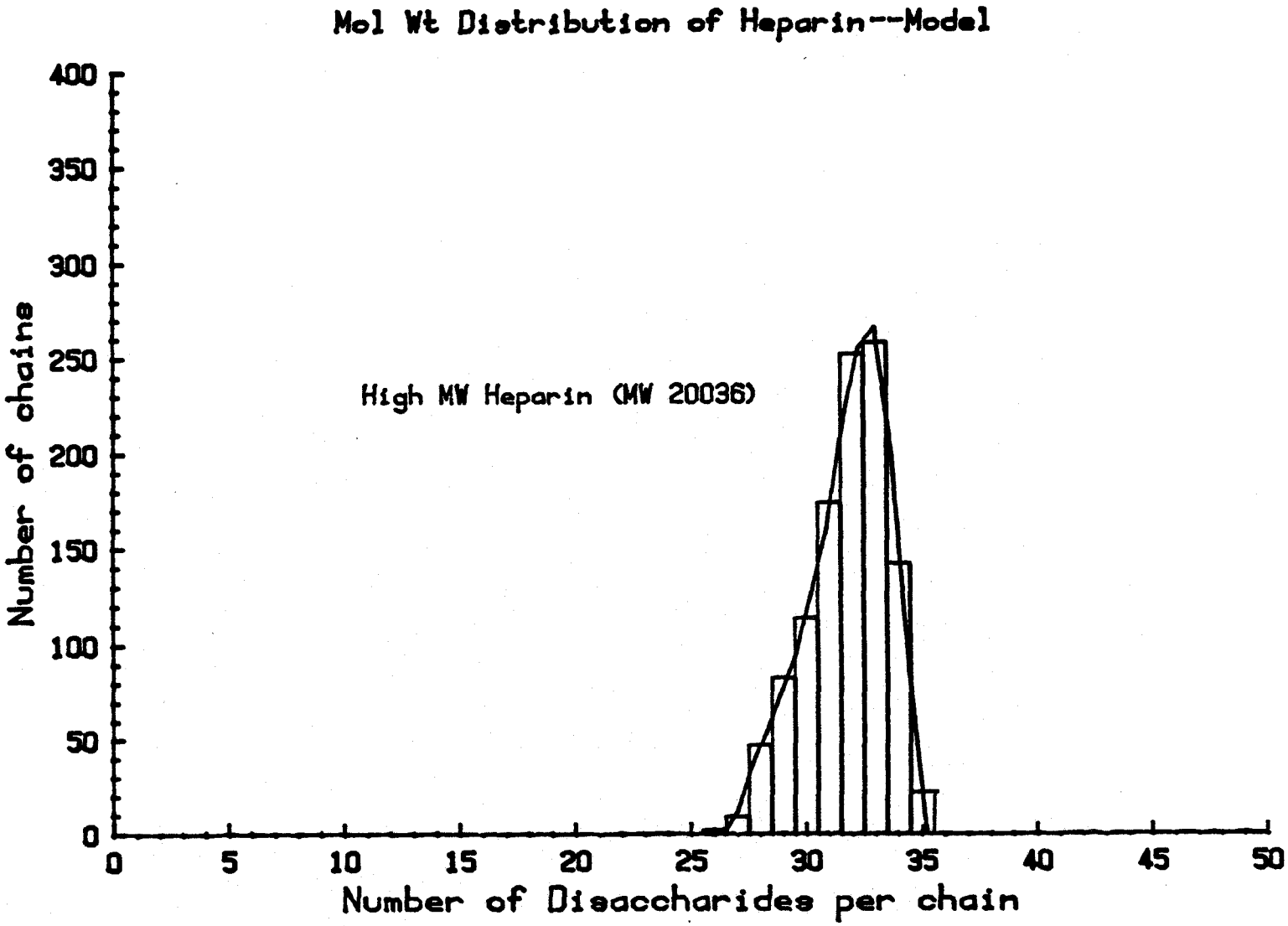
The elution profile for medium low molecular weight heparin presented in Figure 5-2 was converted using Equation 5-1 to show the estimated frequency of each chain length of heparin in medium low molecular weight heparin. The curve shows the relative frequency of each chain length based on the elution pattern of medium low molecular weight heparin. The histogram represents the initial molecular weight distribution of heparin used in the computer simulation of the degradation of medium low molecular weight heparin by heparinase.

FIGURE 6-1E
Mol Wt Distribution of Heparin --Model



The elution profile for medium high molecular weight heparin presented in Figure 5-2 was converted using Equation 5-1 to show the estimated frequency of each chain length of heparin in medium high molecular weight heparin. The curve shows the relative frequency of each chain length based on the elution pattern of medium high molecular weight heparin. The histogram represents the initial molecular weight distribution of heparin used in the computer simulation of the degradation of medium high molecular weight heparin by heparinase.

FIGURE 6-1F



The elution profile for high molecular weight heparin presented in Figure 5-2 was converted using Equation 5-1 to show the estimated frequency of each chain length of heparin in high molecular weight heparin. The curve shows the relative frequency of each chain length based on the elution pattern of high molecular weight heparin. The histogram represents the initial molecular weight distribution of heparin used in the computer simulation of the degradation of high molecular weight heparin by heparinase.

Table 6-1. Initial Distributions of chains used in the model.

Chain Length	Number of chains used					
	Unfract.	Low Mw	M.Low Mw	Med Mw	M.High Mw	High Mw
7	3	0	0	0	0	0
8	10	0	0	0	0	0
9	34	27	0	0	0	0
10	52	182	0	0	0	0
11	54	267	0	0	0	0
12	77	364	0	0	0	0
13	104	390	2	0	0	0
14	113	363	54	0	0	0
15	122	329	162	0	0	0
16	128	279	253	0	0	0
17	129	183	306	0	0	0
18	131	120	341	4	0	0
19	122	46	303	43	0	0
20	113	28	238	64	4	0
21	106	7	170	139	9	0
22	97	1	77	156	37	0
23	87	0	36	204	97	0
24	79	0	17	208	133	0
25	69	0	4	187	164	0
26	58	0	0	161	196	2
27	52	0	0	116	191	9
28	43	0	0	89	178	47
29	37	0	0	43	127	83
30	29	0	0	27	91	114
31	23	0	0	2	48	174
32	21	0	0	0	12	252
33	18	0	0	0	0	258
34	12	0	0	0	0	142
35	11	0	0	0	0	22
36	9	0	0	0	0	0
37	7	0	0	0	0	0
38	6	0	0	0	0	0
39	3	0	0	0	0	0
40	2	0	0	0	0	0
41	1	0	0	0	0	0

This table contains the initial molecular weight distribution assumed for each fraction of heparin and the original sample, expressed as the number of chains of each length used to simulate the initial molecular weight distribution. These data are plotted in Figures 6-1a-f with transformations of the gel permeation elutions for each fraction and the original sample.

molecular weight range tested. Although this information is not necessary to predict the equilibrium distribution of products, it is provided for any worker who should desire to expand the model to predict product distributions throughout the reaction.

CALCULATIONS TO PREDICT THE FINAL PRODUCT DISTRIBUTIONS.

Each of the molecular weight distributions from Table 6-1 and the percent cleavabilities from Table 5-4a were given to a random independent database constructor as described above under Section 3., THEORY. As this method of calculation involves the use of a random parameter, the calculation was repeated many times in order to obtain statistically significant results. The product distributions predicted by the random independent model are shown in Tables 6-2a-f. The first column of numbers is the total number of chains of each particular length predicted by the random independent model. (The numbers in parentheses are the standard deviations based on the number of runs made to produce each table.) The second column is the number of each final product that still has its original non-reducing end, and therefore no chromophore at UV 232nm. The difference between these two columns is the number of each product that have the UV 232nm chromophore. The average differences shown were calculated from the data for each run, and not from the other averages shown. Thus the standard deviations of the first two columns do not add to give the standard deviation of the third column, as the values in the first and second columns are not independent of each other. Finally, the fourth column shows the percent of the total UV 232nm chromophore that the random independent model associates with each product. These percentages can be directly compared to the experimentally measured percentages of each product given in Tables 5-5a-f.

Table 6-2a. Modeled Product Distribution: Random Independent Distribution for Unfractionated Heparin 14 Runs

Product	Total chains	end chains	chains w/chromophore	% of chains w/chromophore
Disaccharide	8225 (30)	860 (10)	7365 (32)	45.64 (.20)
Tetrasaccharide	4443 (24)	461 (7)	3982 (26)	25.16 (.16)
Hexasaccharide	2442 (10)	270 (5)	2172 (12)	13.73 (.08)
Oligosaccharide	2677 (14)	371 (8)	2306 (16)	14.57 (.10)
Total	17787 (62)	1962	15825 (62)	100.00

Legend:

The numbers in parentheses are the standard deviations of each number, based on 14 separate trials of the model.

Total chains denotes the average number of each type of product that the model predicts will be present following total degradation of the 1962 original heparin molecules. Total degradation is defined as degradation of 43.71% of the total alpha linkages.

End chains denotes the average number of each type of chain that still had an original left-handed end. These chains would not be expected to have the UV 232nm chromophore from heparinase.

Chains with chromophore denotes the difference between the first two numbers. The standard deviation is smaller than the sum of the standard deviations because the two events (a chain being a disaccharide and a chain being a disaccharide with an original non-reducing end) are not independent.

The percentages predicted here correspond to the percentages of products obtained by gel permeation chromatography assaying for the UV 232nm chromophore.

Table 6-2b. Modeled Product Distribution: Random Independent Distribution for Low MW Heparin 7 Runs

Product	Total chains	end chains	chains w/chromophore	% of chains w/chromophore
Disaccharide	8173 (35)	1211 (12)	6962 (40)	48.73 (.28)
Tetrasaccharide	4224 (25)	611 (9)	3613 (27)	25.29 (.19)
Hexasaccharide	2360 (14)	343 (7)	2017 (17)	14.12 (.12)
Oligosaccharide	2115 (9)	421 (8)	1694 (13)	11.86 (.09)
Total	17787 (69)	2586	14286 (69)	100.00

Legend:

The numbers in parentheses are the standard deviations of each number, based on 7 separate trials of the model.

Total chains denotes the average number of each type of product that the model predicts will be present following total degradation of the 2586 original heparin molecules. Total degradation is defined as degradation of 43.02% of the total alpha linkages.

End chains denotes the average number of each type of chain that still had an original left-handed end. These chains would not be expected to have the UV 232nm chromophore from heparinase.

Chains with chromophore denotes the difference between the first two numbers. The standard deviation is smaller than the sum of the standard deviations because the two events (a chain being a disaccharide and a chain being a disaccharide with an original non-reducing end) are not independent.

The percentages predicted here correspond to the percentages of products obtained by gel permeation chromatography assaying for the UV 232nm chromophore.

Table 6-2c. Modeled Product Distribution: Random Independent Distribution for Medium Low MW Heparin 7 Runs

Product	Total chains	end chains	chains w/chromophore	% of chains w/chromophore
Disaccharide	7944 (39)	865 (12)	7079 (44)	46.92 (.29)
Tetrasaccharide	4335 (30)	471 (6)	3864 (30)	25.61 (.20)
Hexasaccharide	2281 (14)	256 (5)	2025 (15)	13.42 (.10)
Oligosaccharide	2491 (10)	371 (9)	2120 (15)	14.05 (.10)
Total	17051 (60)	1963	15088 (60)	100.00

Legend:

The numbers in parentheses are the standard deviations of each number, based on 7 separate trials of the model.

Total chains denotes the average number of each type of product that the model predicts will be present following total degradation of the 1963 original heparin molecules. Total degradation is defined as degradation of 44.70% of the total alpha linkages.

End chains denotes the average number of each type of chain that still had an original left-handed end. These chains would not be expected to have the UV 232nm chromophore from heparinase.

Chains with chromophore denotes the difference between the first two numbers. The standard deviation is smaller than the sum of the standard deviations because the two events (a chain being a disaccharide and a chain being a disaccharide with an original non-reducing end) are not independent.

The percentages predicted here correspond to the percentages of products obtained by gel permeation chromatography assaying for the UV 232nm chromophore.

Table 6-2d. Modeled Product Distribution: Random Independent Distribution for Medium MW Heparin 7 Runs

Product	Total chains	end chains	chains w/chromophore	% of chains w/chromophore
Disaccharide	7615 (36)	680 (10)	6935 (41)	47.23 (.28)
Tetrasaccharide	4080 (25)	340 (6)	3740 (28)	25.47 (.19)
Hexasaccharide	2218 (14)	212 (4)	2006 (15)	13.66 (.10)
Oligosaccharide	2214 (14)	211 (6)	2003 (18)	13.64 (.12)
Total	16127 (66)	1443	14684 (66)	100.00

Legend:

The numbers in parentheses are the standard deviations of each number, based on 7 separate trials of the model.

Total chains denotes the average number of each type of product that the model predicts will be present following total degradation of the 1443 original heparin molecules. Total degradation is defined as degradation of 44.01% of the total alpha linkages.

End chains denotes the average number of each type of chain that still had an original left-handed end. These chains would not be expected to have the UV 232nm chromophore from heparinase.

Chains with chromophore denotes the difference between the first two numbers. The standard deviation is smaller than the sum of the standard deviations because the two events (a chain being a disaccharide and a chain being a disaccharide with an original non-reducing end) are not independent.

The percentages predicted here correspond to the percentages of products obtained by gel permeation chromatography assaying for the UV 232nm chromophore.

Table 6-2e. Modeled Product Distribution: Random Independent Distribution for Medium High MW Heparin 7 Runs

Product	Total chains	end chains	chains w/chromophore	% of chains w/chromophore
Disaccharide	7193 (36)	570 (8)	6623 (39)	46.02 (.27)
Tetrasaccharide	3929 (23)	300 (5)	3629 (24)	25.22 (.17)
Hexasaccharide	2151 (16)	182 (4)	1969 (16)	13.68 (.11)
Oligosaccharide	2405 (19)	235 (4)	2170 (19)	15.08 (.13)
Total	15678 (50)	1287	14391 (50)	100.00

Legend:

The numbers in parentheses are the standard deviations of each number, based on 7 separate trials of the model.

Total chains denotes the average number of each type of product that the model predicts will be present following total degradation of the 1287 original heparin molecules. Total degradation is defined as degradation of 43.82% of the total alpha linkages.

End chains denotes the average number of each type of chain that still had an original left-handed end. These chains would not be expected to have the UV 232nm chromophore from heparinase.

Chains with chromophore denotes the difference between the first two numbers. The standard deviation is smaller than the sum of the standard deviations because the two events (a chain being a disaccharide and a chain being a disaccharide with an original non-reducing end) are not independent.

The percentages predicted here correspond to the percentages of products obtained by gel permeation chromatography assaying for the UV 232nm chromophore.

Table 6-2f. Modeled Product Distribution: Random Independent Distribution for High MW Heparin 7 Runs

Product	Total chains	end chains	chains w/chromophore	% of chains w/chromophore
Disaccharide	7186 (33)	467 (7)	6719 (38)	45.87 (.26)
Tetrasaccharide	3885 (20)	247 (5)	3638 (23)	24.84 (.16)
Hexasaccharide	2249 (18)	153 (3)	2096 (19)	14.31 (.13)
Oligosaccharide	2430 (22)	236 (5)	2194 (25)	14.98 (.17)
Total	15750 (46)	1103	14647 (46)	100.00

Legend:

The numbers in parentheses are the standard deviations of each number, based on 7 separate trials of the model.

Total chains denotes the average number of each type of product that the model predicts will be present following total degradation of the 1103 original heparin molecules. Total degradation is defined as degradation of 43.20% of the total alpha linkages.

End chains denotes the average number of each type of chain that still had an original left-handed end. These chains would not be expected to have the UV 232nm chromophore from heparinase.

Chains with chromophore denotes the difference between the first two numbers. The standard deviation is smaller than the sum of the standard deviations because the two events (a chain being a disaccharide and a chain being a disaccharide with an original non-reducing end) are not independent.

The percentages predicted here correspond to the percentages of products obtained by gel permeation chromatography assaying for the UV 232nm chromophore.

The template insertion model was used to assess the effect that the initial distribution of heparinase cleavable alpha linkages has on the final distribution of products. The hexasaccharide sequence '101', representing three adjacent alpha linkages which are cleavable, uncleavable, and cleavable, respectively, was inserted into the initial ensemble of chains to increase its occurrence in the ensemble by 2% without altering the overall percentage of cleavable or uncleavable linkages. The percentage of UV 232nm chromophore attributed to each final product was found by the same calculations used for the random, independent model. The calculations and results for ensembles of chains representing each heparin fraction and the original sample are shown in Tables 6-3a-f.

An attempt was made to measure the effect of the initial distribution of heparinase-cleavable alpha linkages on the final distribution of products using diad and triad analysis. The results obtained from these models are very inconclusive. As was mentioned above in the THEORY section, it is very difficult to control both the amount of dependence and the overall percent of cleavable sites in ensembles of chains constructed using the conditional probabilities of diad and triad analyses. This problem becomes apparent in the very high variation between different trials of the model. The final product distribution predicted by a diad analysis model that is roughly equivalent to a 2% increase in the amount of alternating sequences is given in Table 6-4. A triad analysis model of roughly the same type was used to yield the predictions for the final product distribution shown in Table 6-5. The standard deviations for diad and triad analysis are between 5 and 10 times the size of the standard deviations produced even for fewer runs of either the random independent model or the template insertion model. It is thus very difficult to specify exactly what product distribution these models actually

Table 6-3a. Modeled Product Distribution: Non-Random Independent Distribution for Unfractionated Heparin 10 Runs

Product	Total chains	end chains	chains w/chromophore	% of chains w/chromophore
Disaccharide	8373 (38)	873 (10)	7500 (41)	47.24 (.26)
Tetrasaccharide	4535 (31)	470 (8)	4065 (33)	25.66 (.21)
Hexasaccharide	2359 (15)	260 (6)	2099 (17)	13.22 (.11)
Oligosaccharide	2563 (20)	359 (10)	2204 (24)	13.88 (.15)
Total	17839 (68)	1962	15877 (68)	100.00

Legend:

The numbers in parentheses are the standard deviations of each number, based on 10 separate trials of the template-insertion model.

Total chains denotes the average number of each type of product that the template insertion model predicts will be present following total degradation of the 1962 original heparin molecules used to model unfractionated heparin (see Figure 6-1). The template inserted was the tetrasccharide '101', inserted to increase its frequency by 2%. Total degradation was defined as degradation of 43.71% of the total alpha linkages. End chains denotes the average number of each type of chain that still had an original left-handed (non-reducing) end. These chains would not be expected to have the UV 232nm chromophore from heparinase.

Chains with chromophore denotes the difference between the first two numbers. The standard deviation was smaller than the sum of the standard deviations because the two events (a chain being a disaccharide and a chain being a disaccharide with an original non-reducing end) were not independent.

The percentages predicted here should correspond to the percentages of products obtained by gel permeation chromatography assaying for the UV 232nm chromophore if this template insertion model accurately reflects the distribution of cleavable sites in the original heparin molecules.

Table 6-3b. Modeled Product Distribution: Non-Random Independent Distribution for Low MW Heparin 5 Runs

Product	Total chains	end chains	chains w/chromophore	% of chains w/chromophore
Disaccharide	8294 (37)	1229 (15)	7065 (44)	49.46 (.31)
Tetrasaccharide	4308 (25)	623 (11)	3685 (30)	25.80 (.21)
Hexasaccharide	2312 (17)	328 (10)	1984 (23)	13.89 (.16)
Oligosaccharide	1956 (12)	406 (10)	1550 (17)	10.85 (.12)
Total	16870 (54)	2586	14284 (54)	100.00

Legend:

The numbers in parentheses are the standard deviations of each number, based on 5 separate trials of the template-insertion model.

Total chains denotes the average number of each type of product that the template insertion model predicts will be present following total degradation of the 2586 original heparin molecules used to model unfractionated heparin (see Figure 6-1). The template inserted was the tetrasccharide '101', inserted to increase its frequency by 2%. Total degradation was defined as degradation of 43.02% of the total alpha linkages. End chains denotes the average number of each type of chain that still had an original left-handed (non-reducing) end. These chains would not be expected to have the UV 232nm chromophore from heparinase.

Chains with chromophore denotes the difference between the first two numbers. The standard deviation was smaller than the sum of the standard deviations because the two events (a chain being a disaccharide and a chain being a disaccharide with an original non-reducing end) were not independent.

The percentages predicted here should correspond to the percentages of products obtained by gel permeation chromatography assaying for the UV 232nm chromophore if this template insertion model accurately reflects the distribution of cleavable sites in the original heparin molecules.

Table 6-3c. Modeled Product Distribution: Non-Random Independent Distribution for Medium Low MW Heparin 5 Runs

Product	Total chains	end chains	chains w/chromophore	% of chains w/chromophore
Disaccharide	8104 (41)	878 (15)	7226 (49)	47.62 (.32)
Tetrasaccharide	4445 (30)	482 (8)	3963 (32)	26.12 (.21)
Hexasaccharide	2205 (18)	249 (8)	1956 (21)	12.89 (.14)
Oligosaccharide	2383 (13)	354 (12)	2029 (20)	13.37 (.13)
Total	17137 (82)	1963	15174 (82)	100.00

Legend:

The numbers in parentheses are the standard deviations of each number, based on 5 separate trials of the template-insertion model.

Total chains denotes the average number of each type of product that the template insertion model predicts will be present following total degradation of the 1963 original heparin molecules used to model unfractionated heparin (see Figure 6-1). The template inserted was the tetrasccharide '101', inserted to increase its frequency by 2%. Total degradation was defined as degradation of 44.70% of the total alpha linkages. End chains denotes the average number of each type of chain that still had an original left-handed (non-reducing) end. These chains would not be expected to have the UV 232nm chromophore from heparinase.

Chains with chromophore denotes the difference between the first two numbers. The standard deviation was smaller than the sum of the standard deviations because the two events (a chain being a disaccharide and a chain being a disaccharide with an original non-reducing end) were not independent.

The percentages predicted here should correspond to the percentages of products obtained by gel permeation chromatography assaying for the UV 232nm chromophore if this template insertion model accurately reflects the distribution of cleavable sites in the original heparin molecules.

Table 6-3d. Modeled Product Distribution: Non-Random Independent Distribution for Medium MW Heparin 5 Runs

Product	Total chains	end chains	chains w/chromophore	% of chains w/chromophore
Disaccharide	7718 (37)	687 (12)	7036 (44)	47.94 (.30)
Tetrasaccharide	4159 (29)	349 (8)	3810 (32)	25.98 (.22)
Hexasaccharide	2109 (23)	201 (6)	1908 (22)	13.01 (.15)
Oligosaccharide	2123 (23)	206 (6)	1917 (26)	13.07 (.18)
Total	16109 (85)	1443	14666 (85)	100.00

Legend:

The numbers in parentheses are the standard deviations of each number, based on 5 separate trials of the template-insertion model.

Total chains denotes the average number of each type of product that the template insertion model predicts will be present following total degradation of the 1443 original heparin molecules used to model unfractionated heparin (see Figure 6-1). The template inserted was the tetrasccharide '101', inserted to increase its frequency by 2%. Total degradation was defined as degradation of 44.01% of the total alpha linkages. End chains denotes the average number of each type of chain that still had an original left-handed (non-reducing) end. These chains would not be expected to have the UV 232nm chromophore from heparinase.

Chains with chromophore denotes the difference between the first two numbers. The standard deviation was smaller than the sum of the standard deviations because the two events (a chain being a disaccharide and a chain being a disaccharide with an original non-reducing end) were not independent.

The percentages predicted here should correspond to the percentages of products obtained by gel permeation chromatography assaying for the UV 232nm chromophore if this template insertion model accurately reflects the distribution of cleavable sites in the original heparin molecules.

Table 6-3e. Modeled Product Distribution: Non-Random Independent Distribution for Medium High MW Heparin 5 Runs

Product	Total chains	end chains	chains w/chromophore	% of chains w/chromophore
Disaccharide	7255 (40)	579 (12)	6676 (46)	46.71 (.32)
Tetrasaccharide	3985 (33)	309 (6)	3676 (34)	25.72 (.24)
Hexasaccharide	2169 (18)	209 (6)	1960 (20)	13.71 (.14)
Oligosaccharide	2171 (22)	190 (5)	1981 (24)	13.86 (.17)
Total	15580 (94)	1287	14293 (94)	100.00

Legend:

The numbers in parentheses are the standard deviations of each number, based on 5 separate trials of the template-insertion model.

Total chains denotes the average number of each type of product that the template insertion model predicts will be present following total degradation of the 1287 original heparin molecules used to model unfractionated heparin (see Figure 6-1). The template inserted was the tetrasccharide '101', inserted to increase its frequency by 2%. Total degradation was defined as degradation of 43.82% of the total alpha linkages. End chains denotes the average number of each type of chain that still had an original left-handed (non-reducing) end. These chains would not be expected to have the UV 232nm chromophore from heparinase.

Chains with chromophore denotes the difference between the first two numbers. The standard deviation was smaller than the sum of the standard deviations because the two events (a chain being a disaccharide and a chain being a disaccharide with an original non-reducing end) were not independent.

The percentages predicted here should correspond to the percentages of products obtained by gel permeation chromatography assaying for the UV 232nm chromophore if this template insertion model accurately reflects the distribution of cleavable sites in the original heparin molecules.

Table 6-3f. Modeled Product Distribution: Non-Random Independent Distribution for High MW Heparin 5 Runs

Product	Total chains	end chains	chains w/chromophore	% of chains w/chromophore
Disaccharide	7333 (41)	476 (10)	6857 (47)	46.56 (.32)
Tetrasaccharide	3990 (25)	258 (8)	3732 (28)	25.34 (.19)
Hexasaccharide	2064 (20)	151 (5)	1913 (22)	12.99 (.15)
Oligosaccharide	2443 (22)	218 (8)	2225 (28)	15.11 (.19)
Total	15831 (72)	1103	14728 (72)	100.00

Legend:

The numbers in parentheses are the standard deviations of each number, based on 5 separate trials of the template-insertion model.

Total chains denotes the average number of each type of product that the template insertion model predicts will be present following total degradation of the 1103 original heparin molecules used to model unfractionated heparin (see Figure 6-1). The template inserted was the tetrasccharide '101', inserted to increase its frequency by 2%. Total degradation was defined as degradation of 43.20% of the total alpha linkages. End chains denotes the average number of each type of chain that still had an original left-handed (non-reducing) end. These chains would not be expected to have the UV 232nm chromophore from heparinase.

Chains with chromophore denotes the difference between the first two numbers. The standard deviation was smaller than the sum of the standard deviations because the two events (a chain being a disaccharide and a chain being a disaccharide with an original non-reducing end) were not independent.

The percentages predicted here should correspond to the percentages of products obtained by gel permeation chromatography assaying for the UV 232nm chromophore if this template insertion model accurately reflects the distribution of cleavable sites in the original heparin molecules.

Table 6-4. Modeled Product Distribution: Random Dependent Distribution (based on Diad analysis)

for Unfractionated Heparin 18 Runs

Product	Total chains	end chains	chains w/chromophore	% of chains w/chromophore
Disaccharide	8414 (126)	858 (55)	7556 (152)	47.57 (.96)
Tetrasaccharide	4642 (123)	485 (38)	4157 (141)	26.17 (.89)
Hexasaccharide	2325 (62)	230 (21)	2095 (74)	13.19 (.47)
Oligosaccharide	2464 (78)	389 (31)	2075 (93)	13.06 (.59)
Total	17845 (312)	1962	15883 (312)	100.00

Legend:

The numbers in parentheses are the standard deviations of each number, based on 10 separate trials of the template-insertion model.

Total chains denotes the average number of each type of product that the diad analysis model predicts will be present following total degradation of the 1962 original heparin molecules used to model unfractionated heparin (see Figure 6-1). The probabilities used were:

initiators	prob.	extenders	prob(0)	prob(1)
'00'	.3169	given '00'	.4571	.5429
'01'	.2460	given '01'	.4171	.5829
'10'	.2460	given '10'	.4571	.5429
'11'	.1911	given '11'	.4171	.5829

where 0 represents an uncleavable and 1 a cleavable alpha linkage. Initiators are the first two alpha linkages of the chain. An extender works by the conditional probability that 'if the last two alpha linkages were 00, then the probability that the next alpha linkage will be 0 is .4571.

Total degradation was defined as degradation of 43.71% of the total alpha linkages. End chains denotes the average number of each type of chain that still had an original left-handed (non-reducing) end. These chains would not be expected to have the UV 232nm chromophore from heparinase.

Chains with chromophore denotes the difference between the first two numbers. The standard deviation was smaller than the sum of the standard deviations because the two events (a chain being a disaccharide and a chain being a disaccharide with an original non-reducing end) were not independent.

The percentages predicted here should correspond to the percentages of products obtained by gel permeation chromatography assaying for the UV 232nm chromophore if this diad analysis model accurately reflects the distribution of cleavable sites in the original heparin molecules.

TABLE 6-6a. Comparison of measured product distribution to the product distributions predicted by two different methods.

for Unfractionated heparin

Product	Measured %	Predicted % (random,independent)	Predicted % (template insertion)
Disaccharide	46.77	45.64 (.20)	47.24 (.26)
Tetrasaccharide	25.22	25.16 (.16)	25.66 (.21)
Hexasaccharide	14.00	13.73 (.08)	13.22 (.11)
Oligosaccharide	14.00	14.57 (.10)	13.88 (.15)
		X^2 = .0557 P = 99.52	X^2 = .0593 P = 99.48

TABLE 6-6b. Comparison of measured product distribution to the product distributions predicted by two different methods.

for Low MW Heparin

Product	Measured %	Predicted % (random, independent)	Predicted % (template insertion)
Disaccharide	48.74	48.73 (.28)	49.46 (.31)
Tetrasaccharide	25.19	25.29 (.19)	25.80 (.21)
Hexasaccharide	14.30	14.12 (.12)	13.89 (.16)
Oligosaccharide	11.77	11.86 (.09)	10.85 (.12)
		X^2 = .00337 P = 99.97	X^2 = .1150 P = 99.00

TABLE 6-6c. Comparison of measured product distribution to the product distributions predicted by two different methods.

for Medium Low MW Heparin

Product	Measured %	Predicted % (random,independent)	Predicted % (template insertion)
Disaccharide	46.39	46.92 (.29)	47.62 (.32)
Tetrasaccharide	25.52	25.61 (.20)	26.12 (.21)
Hexasaccharide	13.74	13.42 (.10)	12.89 (.14)
Oligosaccharide	14.35	14.05 (.10)	13.37 (.13)
		X^2 = .0203 P = 99.82	X^2 = .173 P = 98.17

TABLE 6-6d. Comparison of measured product distribution to the product distributions predicted by two different methods.

for Medium MW Heparin

Product	Measured %	Predicted % (random, independent)	Predicted % (template insertion)
Disaccharide	47.78	47.23 (.28)	47.94 (.30)
Tetrasaccharide	25.38	25.47 (.19)	25.98 (.22)
Hexasaccharide	13.98	13.66 (.10)	13.01 (.14)
Oligosaccharide	12.86	13.64 (.12)	13.07 (.13)
		X^2 = .0588 P = 99.49	X^2 = .0901 P = 99.22

TABLE 6-6e. Comparison of measured product distribution to the product distributions predicted by two different methods.

for Medium High MW Heparin

Product	Measured %	Predicted % (random,independent)	Predicted % (template insertion)
Disaccharide	45.99	46.02 (.27)	46.71 (.32)
Tetrasaccharide	26.14	25.22 (.17)	25.72 (.24)
Hexasaccharide	12.88	13.68 (.11)	13.71 (.14)
Oligosaccharide	14.99	15.08 (.13)	13.86 (.17)
		X^2 = .0809 P = 99.30	X^2 = .160 P = 98.36

TABLE 6-6f. Comparison of measured product distribution to the product distributions predicted by two different methods.

for High MW Heparin

Product	Measured %	Predicted % (random,independent)	Predicted % (template insertion)
Disaccharide	45.46	45.87 (.26)	46.56 (.32)
Tetrasaccharide	24.97	24.84 (.16)	25.34 (.19)
Hexasaccharide	14.07	14.31 (.13)	12.99 (.15)
Oligosaccharide	15.50	14.98 (.17)	15.11 (.19)
		X^2 = .0264 P = 99.77	X^2 = .131 P = 98.77

where O_i is the observed frequency of variable i, E_i is the expected frequency of the variable i, and the sum is taken over all variables. Each of the percentages of disaccharide, tetrasaccharide, hexasaccharide, and oligosaccharide were taken as variables. The Chi-square value for the difference between the measured distribution and each predicted distribution is shown in Tables 6-6a-f under the column of the respective predicted distribution. Chi-square values can be used to infer the probability that the difference between two distributions is due to chance. Taking the given Chi-square values and assuming three degrees of freedom (4 variables, 1 equation: the sum is equal to 100%) the probability of the null hypothesis, i.e. the probability that the difference between the measured and predicted distributions is due to chance alone is calculated from the table for the distribution of X^2 given by Downie and Heath[109] and shown below the value for X^2 in Tables 6-6a-f. In every case the probability of the null hypothesis is greater for the random independent model. The template insertion model is off by 1.2% on the average, while the random, independent model is off by only .35% on the average. Thus the assumption of a random, independent initial distribution of heparinase-cleavable sites in the heparin chains leads to a very accurate prediction of the final product distribution. A deviation from this initial distribution of cleavable sites of as little as 2% of a non-random distribution would easily have been distinguished by this method. Almost certainly, therefore, no more than 2% of the cleavable alpha linkages in the heparin molecule are produced by a non-random mechanism.

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109. ibid.

SIGNIFICANCE OF THE RANDOMNESS OF THE CLEAVABLE ALPHA LINKAGES.

The significance of the random distribution of cleavable sites in the heparin molecule lies in its implications toward the structure of heparin. Assuming that the heparinase-cleavable sites are in some unspecified way different chemically from heparinase-uncleavable sites, the random distribution of the cleavable sites implies that at least part of the biosynthesis of heparin is a random process. The specificity of heparinase has been linked to the degree of sulphation and the stereochemistry of the surrounding sugar moieties.[110] This would imply that some aspect of either the degree of sulphation or the stereochemistry of the heparin molecule was randomly produced in the biosynthesis of the heparin chain.

The model used to predict the product distributions also supplied another useful piece of information as a by-product. This was the percentage of each product that will not have the UV 232nm chromophore. For unfractionated heparin, this fraction was about 10% for all of the products. Current research being carried out by both A. Grant of this laboratory and B. Linhardt at the University of Iowa toward purifying the products to determine their structure will be aided knowing that about 20% (10% at the reducing end, 10% at the non-reducing end) of their products will contain endgroups which did not arise from the action of heparinase, but were the original reducing or non-reducing ends of the heparin molecules. Both researchers are collaborating to find the structure of the disaccharide or disaccharides

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110. Linker, A. and P. Hovingh, "Enzymatic Degradation of Heparin as a Tool for Structural Analysis," in Heparin: Structure, Cellular Functions, and Clinical Applications, N.M. McDuffie, ed., New York, Academic Press, 3-38 (1979).

produced by the action of heparinase on heparin. The disaccharide contains information on the structural specificity of the enzyme both at the reducing and non-reducing sides of the alpha linkage. Minor products (amounting to less than 10% of the disaccharide) should not be used to determine the specificity of heparinase, as they represent artifacts from the original heparin preparation.

OPTIMIZATION OF THE IN VIVO REACTOR.

The theoretical studies in the THEORY section concluded that the external reaction model was the only model able to explain the reaction rate observed in the in vivo reactor.[111] This model will now be used to optimize the design of that reactor.

The reactor's current design is shown in Figure 6-2. Fifty ml of Sepharose bead bed is suspended in 100 ml total volume with blood in the reactor. The beads are prevented from leaving the reactor by a nylon mesh screen. The beads are kept in suspension by a peristaltic pump operating at 300 ml/min. Blood from the dog is pumped through the device at a rate of 50 ml/min. The average diameter of the reactor is approximately 6 cm.

The reactor was optimized by varying some parameters of the external reaction model presented in Appendix B and noting the effect on the predicted reaction rate. The model in Appendix B was constructed to make it very easy to modify any combination of design parameters easily. Among the parameters varied were the rate of the fluidization pump, the amount of enzyme used, and the size of the beads. Each of these variables is easily changed experimentally, and could easily be used to check the validity of the external reaction model.

Overall, the external reaction model predicts that the reactor is fairly well optimized, except that the recirculation pump appears to be unnecessary. Doubling the amount of enzyme used in the immobilization from 5.5 mg to 11.0 mg increases the overall reaction rate by only 68%. Considering that the reactor already clears more than 90% of the incoming heparin, increasing the

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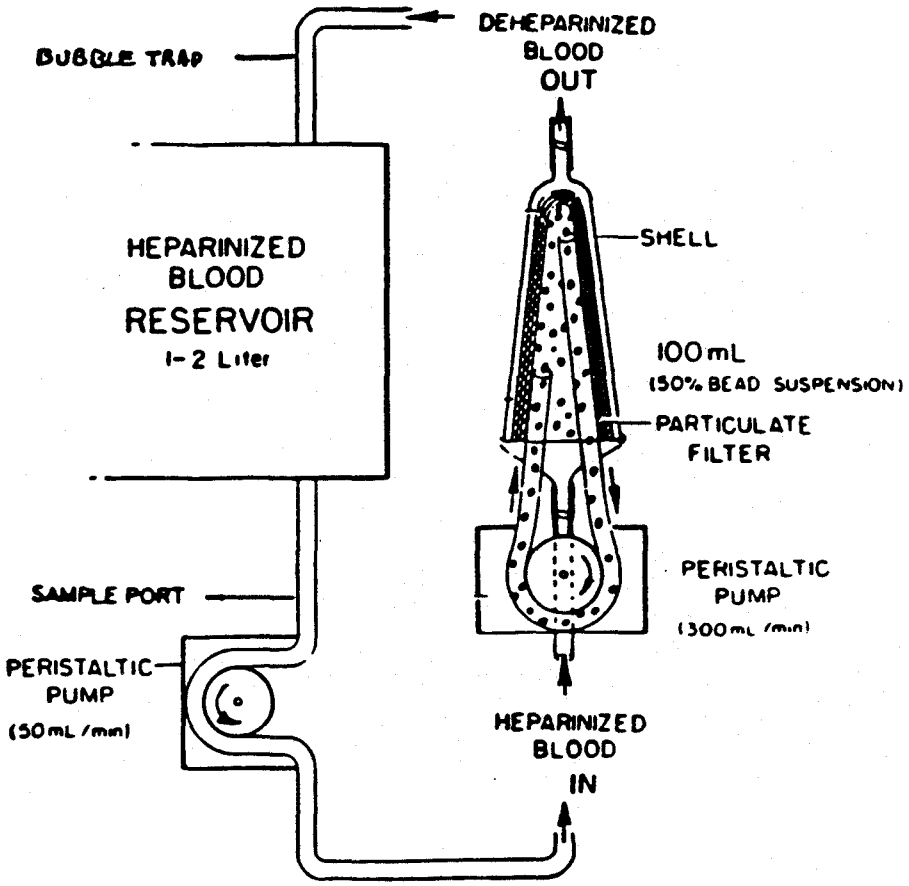
111.Langer, R., R.J. Linhardt, S. Hoffberg, A.K. Larsen, C.L. Cooney, D. Tapper, and M. Klein, "An Enzymatic System for Removing Heparin in Extracorporeal Therapy," Science, 217, 261-263 (1982).



An immobilized heparinase filter.

FIGURE 6-2

amount of enzyme would seem to be a waste of enzyme. Decreasing the amount of enzyme from 5.5 mg to 2.75 mg decreases the reaction rate by 45%, which would not be enough to clear the heparin from the bloodstream. Reducing the size of the beads from 120 microns to 60 microns in diameter, while keeping the total volume of beads constant would increase the reaction rate by 15%. Doubling the diameter of the beads from 120 to 240 microns keeping the total volume of beads constant reduces the reaction rate by 30%. The increase in reaction rate is probably offset by the difficulty of separating the beads from the bloodstream. Smaller beads would be more likely to get through the filter, since the filter pore size must be at least 20 microns across to allow large blood cells to pass through. Doubling the flow rate through the recirculation pump to 600 ml/min gains only a 5% increase in overall reaction rate, due to the weak dependence of the boundary layer thickness around the bead on the fluid velocity. There is also little effect on the rate of reaction if the recirculation pump is eliminated and the fluid velocity inside the reactor is taken as 50 ml/min. The overall reaction rate is reduced only 15%. This loss in overall reaction rate is easily offset by doubling the amount of enzyme as above (overall reaction rate increases 30%) or by halving the diameter of the Sepharose beads used as above (overall reaction rate increases 10%). Perhaps by distributing the inward blood flow to the reactor to have the entering blood flow away from the filter (to keep the beads from clogging the mesh), the 50 ml/min flow rate would be enough to keep the filter clear. The flow through this type of reactor would have to be carefully designed to minimize the amount of blood that passed through the reactor without contacting immobilized heparinase. The recirculation pump is certainly unnecessary from considerations of mass transfer and overall reaction rate.

7. CONCLUSION

Three related investigations of the heparinase-heparin reaction have been conducted. These investigations have shown the sensitivity of the Azure A assay, the effect of immobilization of the enzyme on the reaction rate, and the distribution of the heparinase-cleavable sites in heparin.

Weight-basis standard curves for the Azure A assay were correlated for heparin fractions with different molecular weights. This correlation revealed that the Azure assay is sensitive to the weight of heparin in solution. Thus different preparations of heparin will not produce different results with the Azure A assay within the molecular weight range 5,000 to 40,000 daltons. All that needs to be done to convert the results of the Azure A assay to yield the anticoagulant activity in the solution is to multiply the concentration of heparin given by the assay by the activity of the heparin used. No corrections need to be made for the molecular weight of the heparin sample.

A reactor employing immobilized heparinase to continuously remove heparin from the bloodstream of dogs[112] was modeled using theoretical correlations for the reaction rate and the mass transfer rate from the bulk solution to the surface where the enzyme was immobilized. This mathematical model of the reactor showed that the reactor was efficiently designed, with the exception of a recirculation pump that is not needed to aid mass transfer. The mixing action of the recirculation pump could probably be replaced by using multiple input streams to the reactor. The mathematical model of the reactor was put into the form of a computer program to make the evaluation of any proposed

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112.Langer, R., R.J. Linhardt, S. Hoffberg, A.K. Larsen, C.L. Cooney, D. Tapper, and M. Klein, "An Enzymatic System for Removing Heparin in Extracorporeal Therapy," Science, 217, 261-263 (1982).

design changes simple.

Six different preparations of heparin having different known molecular weight distributions were degraded by soluble heparinase. The molecular weight distribution of the products formed by this reaction was found experimentally using gel permeation chromatography. This distribution of products obtained experimentally was compared with distributions of products formed by computer simulations of heparin degradation that assumed the heparinase-cleavable sites in heparin to be randomly distributed in the heparin molecule. The two distributions were found to be identical to within a 99% confidence limit for all six preparations. Computer simulations of heparin degradation that assumed as little as 2% of a non-random distribution of heparinase-cleavable sites yielded final distributions of products significantly different from the distributions observed experimentally. Assuming that heparinase-cleavable sites are somehow chemically different from uncleavable sites, this implies that there is some particular structure in the heparin molecule that occurs at random. Chemical inspection of the products, such as that currently being done in this laboratory by A. Grant and at the University of Iowa by R. Linhardt, should elucidate this particular structure. Knowing that a particular structure occurs at random will be a great aid in finding the structure of heparin and the reasons for its biological activity.

APPENDIX A

Computer Model of Heparin Degradation by Heparinase

INTENT OF APPENDIX A.

This appendix is intended for programmers wishing to duplicate my calculations used to predict the final product distribution following the degradation of heparin by heparinase. The program presented here contains all of the original BASIC code used to predict the product distribution assuming either a random-independent distribution of cleavable and uncleavable sites or a template insertion model. The program is designed to be used to predict the the product distribution at any given point during the reaction, but was used in this study only for predicting equilibrium distributions.

The program can be easily modified for use given any heparin source. To make this modification easier, certain compilation lists and tables have also been included in this appendix. The program itself is broken into sections, each section containing descriptive comments. The numbers in the first left hand column of each line are the number of statements on the line. Immediately following the listing of the native BASIC code is a list of all the variables used in the program, showing the size and location of the memory allocated to each variable. Following that is a list of all references that the program makes to VAX-11 system routines. This list should make it easier for a programmer to determine if a change in a particular system routine will affect the program. The last two lists are cross-reference lists. The first of these lists all of the line numbers explicitly referenced in the program, each one followed by a list of all the statements that reference that line number (e.g. 630.002 refers to the second statement in line number 630). The second and final list contains all of the variables used in the program in

alphabetical order, followed by the list of all the statements containing that variable. Cross-reference lists make it easier to move sections of a program around or to change variable names. At the end of the appendix is a statement of all of the compilation qualifiers that were in effect when the program was compiled, so that compilation can be exactly duplicated.

AMENDING INPUTS TO THE PROGRAM.

There are seven inputs to the program: (1) the percentage of uncleavable sites, (2) the weight percent and structure of each template to be inserted into the ensemble of chains, (3) the initial chain length distribution of heparin, (4) the relative affinity of heparin for each size of chain, (5) the affinity of heparin for each site of the heparin chain, (6) the number of times that the data base should be analyzed during the simulation and (7) the length of the longest chain in the database. Each of these inputs can be changed to fit any particular measurements and/or assumptions.

The percentage of uncleavable sites and the structure and weight percents of the templates to be inserted are input to the program interactively. The percent of cleavable sites is requested first, in statement 1030.001. Statement 1130.001 requests the structure of the template. A null response is taken as an instruction that no template is to be inserted. A response of "S" is taken as a request to use the same template as was last used. This response is most useful interactively when one is trying to determine an appropriate weight percent for a given template. The weight percent of the particular template that the operator desires is requested at statement 1220.001. At statement 1270.001 the operator is able to request the weight percent be computed on either an overlapping or non-overlapping basis. If the weight percent requested is 0, the program will reply by printing the weight

percent of the template present in a random independent distribution. This interactive method for giving the program templates is very useful for selecting appropriate amounts of the template to insert.

The initial distribution of lengths of the heparin chains is contained in the DATA statements of lines 860-880. The numbers are the number of chains of each length from 1 to 60 inclusive. These numbers are read into the array called SUBSTRATES_OF_LEN% in statement 810.001. The largest chain this program is currently capable of handling is 60 disaccharides in length. This corresponds to a molecular weight of 38,400 daltons. This is much larger than the largest chain in the heparin samples used in this study, which had a maximum molecular weight of about 28,000 daltons. If a larger chain size is desired, the arrays dimensioned in lines 200-280 should be changed, as well as all references to the number 60 in the text of the program. The relative affinity of heparinase for each length of heparin chain is contained in the DATA statements of lines 930-950. The affinity of heparinase for a disaccharide was set equal to 0 and all other lengths of chain were assumed to have an equal affinity for heparinase. These numbers were not important for the present study, as only equilibrium product distributions were calculated. The data were read into the array REL_AFFIN_FOR_SUBSTRATE_LEN in statement 910.001. The number of statistical analyses to be done is determined in statement 3040.001. The final number on the line (20 in this model) is the number of statistical analyses done after the first one. These analyses are equally spaced by percent of reaction. For example, 20 specifies that a statistical analysis of the product distribution be done each time another 5% of the cleavable sites are cleaved. The relative affinities of heparinase for different sites in the heparin chain are assumed to be equal. This is not important for equilibrium distributions, but only for kinetic studies. The

site is chosen in statement 3450.001.

OUTPUT FROM THE MODEL.

The subroutine that statistically analyses the database of chains runs from line 4480 to line 5120. This subroutine also includes a scheme for naming the output files that the analyses are written into. Statements 4850.001 and 4860.001 contain a set of strings which correspond to each percent of reaction that the statistics subroutine will record the database. For example, the statistical analysis after 35% of the cleavable sites in the database had been cleaved would be contained in a file named PROD35.OUT.

The first statistics recorded by the statistics subroutine are the number average, weight average, and Z average molecular weight of the chains in the database. These numbers are printed onto the operator's terminal (or into the operator's log file in the case of batch operation), as an indication of how fast the program is running and how far it has progressed. These statistics are also recorded in their own files as (x,y) pairs where x is the number of cuts inflicted on the database and y is the average molecular weight.

Most of the analysis done by the statistics routine is printed out in tabular form. Each time the subroutine is executed, a table is created containing a statistical abstract of the database of chains. The first column of the table, labeled CH.LEN. is the length of the chain. There are thus 60 rows to the table. The second column, labeled TOTLCHN is the total number of chains of that length in the database. The third column, labeled CBOB is the number of chains of that length or longer in the database. Column 4, labeled TOTLEFT, is the total number of chains in the database that would theoretically contain non-reducing ends from the original heparin sample. The column labeled TOTLRT tabulates the total number of chains in the database

that would theoretically contain reducing ends from the original heparin sample. As the program cannot distinguish left from right on the original chains (and therefore doesn't know a reducing from a non-reducing end) the two distributions of columns 4 and 5 should be very similar . Column 6, labeled TOTSUBS, contains the distribution of lengths of chains containing at least one cleavable alpha linkage. These chains are still considered to be substrates of the enzyme. Column 7, marked TOTPROD, is the size distribution of the chains in the database that contain only uncleavable alpha linkages. These chains are final products of the reaction. Columns 8 and 9 are the distributions of sizes of chains that are final products of the reaction and contain non-reducing and reducing ends respectively from the original heparin sample. Again, these two columns contain essentially the same information, as the program does not distinguish reducing from non-reducing ends (but could do so, depending on the site affinity chosen). The duplication is useful for obtaining more points, and therefore a better statistical estimate of the distribution of the number of chains of each size that contain non-reducing ends from the original heparin sample, and therefore lack the UV 232nm chromophore. This statistical abstract of the database is sufficient for the analysis of gel permeation chromatography data based on the absorbance of the products at UV 232nm.

```
1   10!     HEPARIN DEGRADATION BY HEPARINASE -- THE MODEL (MARK V)
1   20!     COPYRIGHT 1982  GERALD L. FITZGERALD
1
1   30!     THIS PROGRAM HAS THE FOLLOWING OUTLINE:
1
1   40!             SECTION                                          LINE #s
1
1   50!     Dimension Matrices, Format Input/Output                   170 -  280
1   60!     User Defined Functions                                    290 -  740
1   70!     Initial Molecular Weight of Heparin and                   750 -  950
1   80!        Affinity of Heparinase for various Heparin chains
1   90!     User Input Variables                                      960 - 1660
1   100!    Initial Database Creation                                1670 - 2280
1   110!    Mutation of Database (if necessary)                      2290 - 2980
1   120!    Cleaving the Chains                                      2990 - 3950
1   130!    Template Counting Subroutines                            3960 - 4480
1   140!    Statistical Subroutine                                   4490 - 5130
1
1
1
1   150!    This version created November 6, 1982
1   160!    FOR INTERACTIVE USE ONLY - NOT FOR BATCH USE
1
1
1
1
1
1   170!*************************************************************************************************************************
    **********
1
1   180!    DIMENSION MATRICES, FORMAT INPUT/OUTPUT
1
1   190!*************************************************************************************************************************
    ************
1
1   200     DIM SUBSTRATES_OF_LEN%(60), MERS_IN_SUBSTRATES_OF_LEN%(60), PRODUCTS_OF_LEN%(60)
1   210     DIM REL_AFFIN_FOR_SUBSTRATE_LEN(60), ABS_AFFIN_FOR_SUBSTRATE_LEN(60)
1   220     DIM TEMPLATE$(5), JUICE_PROB_TEMPLATE(5)
1   230     DIM SUBSTRATE_LEN_SEL_INDEX(60), CHAINS_AS_BIG_OR_BIGGER%(60)
1   240     DIM LEFT_PROD_OF_LEN%(60), RT_PROD_OF_LEN%(60),  LEFT_CHAIN_OF_LEN%(60), RT_CHAIN_OF_LEN%(60)
1   250     DIM ADD_SEQ_NO$(60), COUNT_OVER(5%), SKIPPY%(5%), POSSIBLE(5,60), PROB_OF_MUTATION_TO_LEN(5,60)
1   260     DIM MUTATION_SEL_INDEX(5,60), CHANCES_OF_CHOOSING_LEN(60), TOTAL_CHAINS_OF_LEN%(60%)
1   270     DIM TREE_SEQ$(60),EXT_TEMP$(650), INT_TEMP$(130)
1   280     MAP (TANK) STORAGE$=60%, ORIGEN$=2%
1
1
1
1
1
1   290!*************************************************************************************************************************
    ***********
1
1   300!    USER DEFINED FUNCTIONS
1
1   310!*************************************************************************************************************************
    ***********
1
```

```
1   320     DEF FN.RAND(A,B)        ! RETURNS RANDOM INTEGER BETWEEN A & B INCLUSIVE
1   330             RANDOMIZE       ! A & B ARE INTEGERS, A < B, OTHERWISE, A FATAL ERROR IS SIGNALLED
3   340             IF (A <> INT(A)) OR (B <> INT(B)) THEN PRINT 'ILLEGAL ARGUMENTS -- FN.RAND'\STOP
3   350             IF B < A THEN PRINT 'ILLEGAL ARGUMENTS -- FN.RAND'\STOP
1   360             FN.RAND = A + INT( (B-A+1)* RND)
1   370     FNEND
1
1
1
1
1
1   380     DEF FN.NUMB%(A$,B$)     ! RETURNS NUMBER OF TIMES B$ OCCURS IN A$
1   390             TO% = 0         ! INCLUDES OVERLAPPING OF B$ IN A$
1   400             FOR T% = 1 TO (LEN(A$) - LEN(B$) + 1)
2   410                     IF SEG$(A$, T%, (T% + LEN(B$) -1) ) = B$ THEN TO% = TO% + 1
1   420             NEXT T%
1   430             FN.NUMB% = TO%
1   440     FNEND
1
1
1
1
1
1   450     DEF FN.CLEAVABLE(A$)    ! RETURNS 0 IF A$ IS UNCLEAV, 1 IF CLEAV.
1   460             A = 0           ! or RETURNS 0 IF A$ CONTAINS ONLY '0's, OTHERWISE, RETURNS 1
1   470             WHILE LEN(A$) > 0
1   480                     A = VAL( SEG$( A$, 1, 10)) + A
1   490                     A$ = SEG$(A$, 11, LEN(A$))
1   500             NEXT
3   510             IF A = 0 THEN FN.CLEAVABLE = 0 ELSE FN.CLEAVABLE = 1
1   520     FNEND
1
1
1
1
1
1   530     DEF FN.CORR(A$)         ! RETURNS THE EXPECTED NUMBER OF OCCURENCES (INCLUDING OVERLAPS)
1   540                             !  OF A$ IN THE DISTRIBUTION GIVEN IN THE MODEL
1   550             C = 0           ! C CONTAINS THE NUMBER OF POSSIBLE POSITIONS TO START A$
1   560             FOR A% = (LEN(A$) + 1) TO 60
1   570                     C = C + SUBSTRATES_OF_LEN%(A%) * (A% - LEN(A$))
1   580             NEXT A%
1   590             FN.CORR = C * OVERALL_UNCLEAV^(FN.NUMB%(A$,'0')) * OVERALL_CLEAV^(FN.NUMB%(A$,'1'))
1   600     FNEND
1
1
1
1
1
1   610     DEF FN.TIMES$(A$,A)     ! RETURNS A STRING WHERE A$ HAS BEEN REPEATED A TIMES
2   620             B = 1 \ B$ = ""
2   630             IF A <> INT(A) THEN GOTO 670
1   640             WHILE B <= A
2   650                     B$ = A$ + B$  \  B = B + 1
1   660             NEXT
1   670             FN.TIMES$ = B$
1   680     FNEND
```

```
690     DEF FN.MAX(A,B)         ! RETURNS THE LARGER NUMBER A OR B
700             IF A > B THEN FN.MAX = A ELSE FN.MAX = B
710     FNEND


720     DEF FN.MIN(A,B)         ! RETURNS THE SMALLER NUMBER A OR B
730             IF A < B THEN FN.MIN = A ELSE FN.MIN = B
740     FNEND


750!*************************************************************************************************************************
***********

760!    INITIAL MOLECULAR WEIGHT OF HEPARIN AND RELATIVE AFFINITY OF HEPARINASE FOR DIFFERENT SIZES OF HEPARIN

770!*************************************************************************************************************************
***********

780!    Chain of length N has N disaccharides and N-1 bonds
790     MOL.WT_DISACCH = 640
800     FOR I% = 1 TO 60
810             READ SUBSTRATES_OF_LEN%(I%)
820             SUBSTRATES_OF_LEN%(0%) = SUBSTRATES_OF_LEN%(0%) + SUBSTRATES_OF_LEN%(I%)
830             MERS_IN_SUBSTRATES_OF_LEN%(I%) = I% * SUBSTRATES_OF_LEN%(I%)
840             MERS_IN_SUBSTRATES_OF_LEN%(0%) = MERS_IN_SUBSTRATES_OF_LEN%(0%) + MERS_IN_SUBSTRATES_OF_LEN%(I%)
850     NEXT I%
860     DATA   0,  0,  0,  0,  0,  0,  3, 10, 34, 52, 54, 77,104,113,122,128,129,131,122,113
870     DATA 106, 97, 87, 79, 69, 58, 52, 43, 37, 29, 23, 21, 18, 12, 11,  9,  7,  6,  3,  2
880     DATA   1,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0


890!    Relative Affinities
900     FOR I% = 1 TO 60
910             READ REL_AFFIN_FOR_SUBSTRATE_LEN(I%)
920     NEXT I%
930     DATA 0, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1
940     DATA 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1
950     DATA 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1
```

```
1  960!**********************************************************************************************************************
1  ***********
1
1  970!    USER INPUT VARIABLES:
1  980!            % of alpha linkages which should be cleaved
1  990!            any template that should be added at higher than normal frequency ("juiced")
1  1000!                   the amount of that template that should be added
1  1010!                   whether to count overlaps of that template
1
1  1020!*********************************************************************************************************************
1  ************
1
1  1030    INPUT 'Percent Uncleavable Sites  (0 < N < 100) ' ; OVERALL_UNCLEAV
1
1  1040!   Compute how many bonds are cleavable, how many are uncleavable
1  1050    OVERALL_UNCLEAV = OVERALL_UNCLEAV / 100
1  1060    OVERALL_CLEAV = 1 - OVERALL_UNCLEAV
1  1070    NUMBER_UNCLEAV = OVERALL_UNCLEAV * (MERS_IN_SUBSTRATES_OF_LEN%(0%) - SUBSTRATES_OF_LEN%(0%))
1  1080    NUMBER_CLEAV = OVERALL_CLEAV * (MERS_IN_SUBSTRATES_OF_LEN%(0%) - SUBSTRATES_OF_LEN%(0%))
1
1
1
1
1
1  1090!   Template input and juicing
1  1100    FOR I% = 1 TO 5
1  1110            PRINT
1  1120            PRINT '    Template #';I%;' as binary string, 0 = uncleav.'
1
1  1130            INPUT YODA$
1
2  1140            IF YODA$ = 's' THEN YODA$ = 'S'
2  1150            IF YODA$ = 'S' THEN PRINT TEMPLATE$(I%)
2  1160            IF YODA$ = 'S' THEN GOTO 1190
2  1170            IF YODA$ = '' THEN GOTO 1620
1  1180            TEMPLATE$(I%) = YODA$
1  1190            MAT ADD_SEQ_NO$ = NUL$
1  1200            CONTENT_NO% = 0
1  1210!           Compute the desired occurence frequency.
1
1  1220            INPUT ' Wt. percent of template  (0 < N < 100) ',TEMPLATE_WT
1
1  1230            TEMPLATE_WT = TEMPLATE_WT / 100
1  1240            DESIRED_OCC = (MERS_IN_SUBSTRATES_OF_LEN%(0%) - SUBSTRATES_OF_LEN%(0%)) * TEMPLATE_WT / LEN(TEMPLATE$(I%))
1  1250            RANDOM_OCC = FN.CORR(TEMPLATE$(I%))
1  1260            SKIP_P% = 5%
1
1  1270            INPUT "Allow template to overlap";SKIP$
1
2  1280            IF SKIP$ = "Y" THEN SKIP_P% = 0
2  1290            IF SKIP$ = "N" THEN SKIP_P% = 1
1  1300            SKIPPY%(I%) = SKIP_P%
3  1310            IF SKIP_P% = 5% THEN PRINT "Please Reply Y or N"\GOTO 1260
3
3
3
3
```

```
3
2  1320          IF SKIPPY%(I%) = 0% THEN GOTO 1450
1  1330!         Detect sequences which can overlap themselves, SUBR 3990 & SUBR 4040 to correct the occurence frequency.
1  1340!         Generate the overlapping sequences from longest to shortest.
1  1350          OVERLAP_TRAP = 0
1  1360          LONG% = LEN(TEMPLATE$(I%))
1  1370          OVERLAP_LEN = 1%
1  1380          WHILE OVERLAP_LEN < LONG%
1  1390                FIRST_PART$ = SEG$(TEMPLATE$(I%),1,OVERLAP_LEN)
1  1400                SECOND_PART$ = SEG$( TEMPLATE$(I%), LONG%-OVERLAP_LEN+1, LONG%)
3  1410                IF FIRST_PART$ = SECOND_PART$ THEN OVERLAP_TRAP = 1 \ GOSUB 3980
1  1420                OVERLAP_LEN = OVERLAP_LEN + 1
1  1430          NEXT
2  1440          IF OVERLAP_TRAP = 1 THEN GOSUB 4030
2
2
2
2
2
1  1450          JUICE = DESIRED_OCC - RANDOM_OCC
2  1460          IF JUICE > 0 THEN GOTO 1550     !   Successful Positive Juicing
2  1470          IF DESIRED_OCC <> 0 THEN GOTO 1530      !   "Successful" Negative Juicing
1  1480                PRINT ' Request for information -- Template occurs randomly in ';
1  1490                PRINT 100 * RANDOM_OCC * LEN(TEMPLATE$(I%)) / (MERS_IN_SUBSTRATES_OF_LEN%(0%) - SUBSTRATES_OF_LEN%(0%))
1  1500                PRINT "Please enter another template or 'S' for same"
1  1510                PRINT
1  1520                GOTO 1120
1
1
1  1530!         Compute the inverse juicing to be done.
3  1540          PRINT "*** NEGATIVE JUICING DISALLOWED ***" \ PRINT ' NON-FATAL ERROR -- Template occurs randomly in '; \GOTO 1490
3
3
1  1550!         Compute the juice probabilities and check for overspecification
1  1560          JUICE_PROB_TEMPLATE(I%) = JUICE
1  1570          RANDOM_OCC_TEMPLATE(I%) = RANDOM_OCC
1  1580          DESIRED_OCC_TEMPLATE(I%) = DESIRED_OCC
1  1590          NUMBER_UNCLEAV = NUMBER_UNCLEAV - JUICE * FN.NUMB%(TEMPLATE$(I%), '0')
1  1600          NUMBER_CLEAV = NUMBER_CLEAV - JUICE * FN.NUMB%(TEMPLATE$(I%), '1')
3  1610          IF (NUMBER_UNCLEAV < 0) OR (NUMBER_CLEAV < 0) THEN PRINT 'FATAL ERROR -- OVERSPECIFIED TEMPLATES' \ STOP
1  1620    NEXT I%
1
1
1
1  1630!   Compute probability of Cleavability outside of the templates.
1  1640    NUMBER_FREE = NUMBER_UNCLEAV + NUMBER_CLEAV
1  1650    PROB_FREE_0 = NUMBER_UNCLEAV / NUMBER_FREE
1  1660    PROB_FREE_1 = NUMBER_CLEAV / NUMBER_FREE
1
1
1
1
1  1670!*****************************************************************************************************************************
1  **********
1
1  1680!   CREATION OF INITIAL DATABASE
```

```
1
1  1690!*****************************************************************************************************************************
1  ***********
1
1  1700!   Create Initial Database
1  1710    PRINT
1  1720    PRINT 'INITIALISING DATABASE -- PLEASE WAIT'
1  1730    TOTAL_CLEAV_SITES% = 0%
1  1740    I% = 60                                 !COUNTING DOWN FROM LENGTH 60
1  1750    RANDOMIZE                               !There is great disorder under heaven and the situation is excellent
1  1760    WHILE I% > 0
1  1770    FILENAME$ = 'DRB1:[GERRY.MODELS]BASE' + STR$(I%) + '.DAT'
1  1780    OPEN FILENAME$ AS FILE #1% , RELATIVE , ACCESS MODIFY , MAP TANK
1  1790            J% = SUBSTRATES_OF_LEN%(I%)             !COUNTING DOWN # OF SUBSTRATES TO BE MADE
1  1800            CHAIN$=''                               !INITIALISING THE CHAIN AS EMPTY
1  1810            ORIGEN$="LR"
1  1820            MAT COUNT_OVER = ZER
1  1830            WHILE J% > 0
2  1840                    IF LEN(CHAIN$) = (I% - 1) THEN GOTO 1990  !KICK OUT FINISHED CHAINS
2
2
2
2
2
1  1850!                   Assume Cleavable sites are independent
1  1860                    COINFLIP = RND
3  1870                    IF COINFLIP <= PROB_FREE_0 THEN TEMPLATE$(0%) = '0' ELSE TEMPLATE$(0%) = '1'
1  1880                    CHAIN$ = CHAIN$ + TEMPLATE$(0%)
2  1890                    IF TEMPLATE$(0%) = '1' THEN TOTAL_CLEAV_SITES% =  TOTAL_CLEAV_SITES% + 1
2
2
2
2
2
1  1900                    OUT_TO = LEN(CHAIN$)
1  1910                    FOR K% = 1 TO 5
2  1920                            IF TEMPLATE$(K%) = "" THEN GOTO 1970
3  1930                            IF COUNT_OVER(K%) > 0 THEN COUNT_OVER(K%) = COUNT_OVER(K%) - 1 \ GOTO 1970
2  1940                            IF TEMPLATE$(K%) <> SEG$( CHAIN$, OUT_TO + 1 - LEN(TEMPLATE$(K%)), OUT_TO ) THEN GOTO 1970
1  1950                            TEMPLATE_NUMBER%(K%) = TEMPLATE_NUMBER%(K%) + 1%
2  1960                            IF SKIPPY%(K%) = 1 THEN COUNT_OVER(K%) = LEN(TEMPLATE$(K%)) - 1
1  1970                    NEXT K%
1
1
1  1980                    GOTO 1840 ! Test if chain is finished and continue on production loop
1
1
1
1
1
1  1990                    ! CHAIN FINISHED
1  2000                    LEFT_CHAIN_OF_LEN%(I%) = LEFT_CHAIN_OF_LEN%(I%) + 1
1  2010                    RT_CHAIN_OF_LEN%(I%) = RT_CHAIN_OF_LEN%(I%) + 1
1  2020                    LEFT_CHAIN_OF_LEN%(0%) = LEFT_CHAIN_OF_LEN%(0%) + 1
1  2030                    RT_CHAIN_OF_LEN%(0%) = RT_CHAIN_OF_LEN%(0%) + 1
2  2040                    IF FN.CLEAVABLE(CHAIN$) <> 0 THEN GOTO 2140
1  2050                    PRODUCTS_OF_LEN%(I%) = PRODUCTS_OF_LEN%(I%) + 1
```

```
1  2060                 SUBSTRATES_OF_LEN%(I%) = SUBSTRATES_OF_LEN%(I%) - 1
1  2070                 LEFT_PROD_OF_LEN%(I%) = LEFT_PROD_OF_LEN%(I%) + 1
1  2080                 RT_PROD_OF_LEN%(I%) = RT_PROD_OF_LEN%(I%) + 1
1  2090                 PRODUCTS_OF_LEN%(0%) = PRODUCTS_OF_LEN%(0%) + 1
1  2100                 SUBSTRATES_OF_LEN%(0%) = SUBSTRATES_OF_LEN%(0%) - 1
1  2110                 LEFT_PROD_OF_LEN%(0%) = LEFT_PROD_OF_LEN%(0%) + 1
1  2120                 RT_PROD_OF_LEN%(0%) = RT_PROD_OF_LEN%(0%) + 1
1  2130                 GOTO 2160
1  2140                 STORAGE$ = CHAIN$
1  2150                 PUT #1%
1  2160                 CHAIN$ = ''  ! REINITIALIZE
1  2170                 MAT COUNT_OVER = ZER
1  2180                 ORIGEN$ = "LR"
1  2190                 J% = J% - 1
1  2200           NEXT
1  2210           I% = I% -1
1  2220           CLOSE #1%
1  2230    NEXT
1
1
1
1  2240    GOSUB 4510! Do initial statistical counts
1
1
1
1  2250    FOR I% = 1% TO 5%
1  2260           JUICE_OCC_TEMPLATE(I%) = INT( FN.MAX( (DESIRED_OCC_TEMPLATE(I%) - TEMPLATE_NUMBER%(I%)), 0 ) )
2  2270           IF TEMPLATE$(I%) <> "" THEN PRINT "Template #";I%;" occurs ";TEMPLATE_NUMBER%(I%);" times." &
3                                         \ PRINT "This compares with an expectation of ";RANDOM_OCC_TEMPLATE(I%);" times." &
4                                         \ PRINT "In addition, ";JUICE_OCC_TEMPLATE(I%) ;" will be added."
1  2280    NEXT I%
1
1
1
1
1
1  2290!*****************************************************************************************************************
1  ***********
1
1  2300!   MUTATION OF DATABASE (IF NECESSARY)
1
1  2310!*****************************************************************************************************************
1  ***********
1
1  2320!   Inform operator of status
1  2330    PRINT
1  2340    PRINT "DATA BASE BEING MUTATED WITH TEMPLATES -- ONE MOMENT PLEASE"
1  2350    PRINT
1
1
1  2360!   Count the number of possible places that the template can be
1  2370    MAT POSSIBLE = ZER
1  2380    FOR I% = 1 TO 5
2  2390           IF TEMPLATE$(I%) = "" THEN GOTO 2450
1  2400           FOR J% = (LEN(TEMPLATE$(I%)) + 1) TO 60
1  2410                  POSSIBLE(I%,J%) = POSSIBLE(I%,J%) + SUBSTRATES_OF_LEN%(J%) * (J% - LEN(TEMPLATE$(I%)))
1  2420                  POSSIBLE(I%,0%) = POSSIBLE(I%,0%) + SUBSTRATES_OF_LEN%(J%) * (J% - LEN(TEMPLATE$(I%)))
```

```
1  2430          NEXT J%
1  2440!  Randomly choose a chain to be mutated
1  2450   NEXT I%
1
1
1
1  2460!  Compute the probability of a given chain length to be selected for mutation
1  2470   MAT PROB_OF_MUTATION_TO_LEN = ZER
1  2480   MAT MUTATION_SEL_INDEX = ZER
1  2490   FOR I% = 1 TO 5
2  2500           IF TEMPLATE$(I%) = "" THEN GOTO 2550
1  2510           FOR J% = 1 TO 60
1  2520                   PROB_OF_MUTATION_TO_LEN(I%,J%) = POSSIBLE(I%,J%) / POSSIBLE(I%,0%)
1  2530                   MUTATION_SEL_INDEX(I%,J%) = MUTATION_SEL_INDEX(I%,J%- 1) + PROB_OF_MUTATION_TO_LEN(I%,J%)
1  2540           NEXT J%
1  2550   NEXT I%
1
1
1
1  2560   RANDOMIZE                              !There is great disorder under heaven and the situation is excellent.
1  2570   FOR I% = 1 TO 5                        !Select a length of chain to be mutated
2  2580           IF TEMPLATE$(I%) = ""  THEN GOTO 2940
2  2590           IF JUICE_OCC_TEMPLATE(I%) < 0 THEN GOTO 2940
1  2600           FOR J% = 1 TO JUICE_OCC_TEMPLATE(I%)
1  2610                   COINFLIP = RND
1  2620                   K = 1
1  2630                   FOR L% = 1 TO 60
2  2640                           IF COINFLIP > MUTATION_SEL_INDEX(I%,L%) THEN K = L% + 1
1  2650                   NEXT L%
1  2660                   CHOSEN_LENGTH% = K
2  2670                   IF K = 1 THEN GOTO 2610
2
2
2
1  2680!                  Retrieve the chain from the database
1  2690                   FILENAME$ = 'DRB1:[GERRY.MODELS]BASE' + STR$(CHOSEN_LENGTH%) + '.DAT'
1  2700                   OPEN FILENAME$ AS FILE #1%, RELATIVE, ACCESS MODIFY, MAP TANK
1  2710                   CHOSEN_SUBSTRATE% = FN.RAND(1, SUBSTRATES_OF_LEN%(CHOSEN_LENGTH%))      ! Pick a SUBSTRATE
1  2720                   GET #1%, RECORD CHOSEN_SUBSTRATE%                                       ! Get Chosen SUBSTRATE from storage
1  2730                   CHOSEN_STRING$ = TRM$(STORAGE$)                                         ! Trim stored version for use
1  2740                   CHOSEN_SITE% = FN.RAND( 1, (LEN(CHOSEN_STRING$) - LEN(TEMPLATE$(I%)) + 1)) &
2                         \       ! Choose the mutation site
1  2750                   CHOSEN_PLACE$ = SEG$(CHOSEN_STRING$, CHOSEN_SITE%, CHOSEN_SITE% + LEN(TEMPLATE$(I%)) - 1)
1
1  2760!  Determine if the template is in the chosen position
1  2770!  If overlaping templates matter, see if the template overlaps with the chosen position
1  2780!  If no template will be destroyed, then insert the template in the given position, else choose another site
1  2790                   FOR L = (CHOSEN_SITE% - LEN(TEMPLATE$(I%)) + 1) TO (CHOSEN_SITE% +  LEN(TEMPLATE$(I%)) - 1)
2  2800   IF TEMPLATE$(I%) <> SEG$(CHOSEN_STRING$, L, L + LEN(TEMPLATE$(I%)) - 1) THEN GOTO 2850
1  2810   PLACE_OVER$ = SEG$(CHOSEN_STRING$, FN.MAX(L,CHOSEN_SITE%),FN.MIN(L,CHOSEN_SITE%)+LEN(TEMPLATE$(I%)) - 1)
1  2820   OVER_PLACE$ = SEG$(TEMPLATE$(I%),FN.MAX(1,L-CHOSEN_SITE%),FN.MIN((L+LEN(TEMPLATE$(I%))-CHOSEN_SITE%),LEN(TEMPLATE$(
1  I%))))
2  2830   IF (OVER_PLACE$ = PLACE_OVER$) AND SKIPPY(I%) = 0% THEN GOTO 2850
2  2840   CLOSE #1% \ GOTO 2610
1  2850                   NEXT L
1
1  2860!                  Correct the counts of cleavable sites and update the database.
```

```
1  2870                 TOTAL_CLEAV_SITES% = TOTAL_CLEAV_SITES% - FN.NUMB%(CHOSEN_PLACE$ , '1')
1  2880                 TOTAL_CLEAV_SITES% = TOTAL_CLEAV_SITES% + FN.NUMB%(TEMPLATE$(I%) , '1')
1  2890                 IN1$ = SEG$(CHOSEN_STRING$, 1%, (CHOSEN_SITE% - 1%))
1  2900                 IN2$ = SEG$(CHOSEN_STRING$, (CHOSEN_SITE% + LEN(TEMPLATE$(I%))), LEN(CHOSEN_STRING$))
1  2910                 OUT$ = IN1$ + TEMPLATE$(I%) + IN2$
2  2920                 UPDATE #1% \ CLOSE #1%
1  2930             NEXT J%
1  2940     NEXT I%
1
1
1  2950     PRINT TOTAL_CLEAV_SITES%;" Cleavable sites in this database."
1  2960     PRINT OVERALL_CLEAV * (MERS_IN_SUBSTRATES_OF_LEN%(0%) - SUBSTRATES_OF_LEN%(0%));" Sites were expected."
1  2970     PRINT
1
1
1
1  2980     PRINT 'INITIAL DATABASE FINISHED'
1
1
1
1  29901**********************************************************************************************************************
1  ***********
1
1  30001    CLEAVING THE CHAINS
1
1  30101**********************************************************************************************************************
1  ***********
1
1  30201    Compute chances for the chains to get cut.
1  3030     RANDOMIZE                              !There is great disorder under heaven and the situation is excellent.
1  3040     DO_A_COUNT_EVERY = INT(TOTAL_CLEAV_SITES% / 20)
1  3050     MAT CHANCES_OF_CHOOSING_LEN = ZER
1  3060         CHANCES_OF_CHOOSING_LEN(0%) = 0
1  3070     MAT SUBSTRATE_LEN_SEL_INDEX = ZER
1  3080         SUBSTRATE_LEN_SEL_INDEX(0%) = 0
1  3090     FOR I% = 1 TO 60
1  3100             CHANCES_OF_CHOOSING_LEN(I%) = REL_AFFIN_FOR_SUBSTRATE_LEN(I%) * SUBSTRATES_OF_LEN%(I%)
1  3110             CHANCES_OF_CHOOSING_LEN(0%) = CHANCES_OF_CHOOSING_LEN(0%) + CHANCES_OF_CHOOSING_LEN(I%)
1  3120     NEXT I%
4  3130     IF CHANCES_OF_CHOOSING_LEN(0%) = 0% THEN TOTAL_CLEAV_SITES% = CUTS \ GOSUB 4530\ GOTO 5130
4
1  31401    Take the affinity for substrates into account
1  3150     FOR I% = 1 TO 60
1  3160             ABS_AFFIN_FOR_SUBSTRATE_LEN(I%) = CHANCES_OF_CHOOSING_LEN(I%) / CHANCES_OF_CHOOSING_LEN(0%)
1  3170             SUBSTRATE_LEN_SEL_INDEX(I%) = SUBSTRATE_LEN_SEL_INDEX(I% - 1) +  ABS_AFFIN_FOR_SUBSTRATE_LEN(I%)
1  3180     NEXT I%
1
1
1
1  31901    Choose length of chain to be cut.
1  3200     COIN_FLIP = RND
1  3210     K% = 1
1  3220     FOR I% = 1 TO 60
2  3230             IF COIN_FLIP > SUBSTRATE_LEN_SEL_INDEX(I%) THEN K% = I% + 1
1  3240     NEXT I%
```

```
2  3250    IF K% = 1 THEN GOTO 3200
1  3260    CHOSEN_LENGTH% = K%
1
1
1
1
1
1  3270!   Extract SUBSTRATE from database
1  3280    FILENAME$ = 'DRB1:[GERRY.MODELS]BASE' + STR$(CHOSEN_LENGTH%) + '.DAT'
1  3290    OPEN FILENAME$ AS FILE #1%, RELATIVE, ACCESS MODIFY, MAP TANK
1  3300    CHOSEN_SUBSTRATE% = FN.RAND(1, SUBSTRATES_OF_LEN%(CHOSEN_LENGTH%))      ! Pick a SUBSTRATE
1  3310    GET #1%, RECORD CHOSEN_SUBSTRATE%                                       ! Get Chosen SUBSTRATE from storage
1  3320    DELETE #1%                                                  ! Delete it from the database
1  3330    CHOSEN_STRING$ = TRM$(STORAGE$)                             ! Trim stored version for use
1  3340    PLUG$ = ORIGEN$
2  3350    IF CHOSEN_SUBSTRATE% = SUBSTRATES_OF_LEN%(CHOSEN_LENGTH%) THEN GOTO 3400 ! If last record was deleted, don't do it
2  again
1  3360    GET #1%, RECORD SUBSTRATES_OF_LEN%(CHOSEN_LENGTH%)                      ! Get last record in file
1  3370    DELETE #1%                                                  ! Delete from that position
1  3380    PUT #1%, RECORD CHOSEN_SUBSTRATE%                                       ! Insert it in the hole
1  3390    CLOSE #1%
1  3400    SUBSTRATES_OF_LEN%(CHOSEN_LENGTH%) = SUBSTRATES_OF_LEN%(CHOSEN_LENGTH%) - 1 ! Decrement tally of that SUBSTRATE len
1  gth
1  3410    SUBSTRATES_OF_LEN%(0%) = SUBSTRATES_OF_LEN%(0%) + 1                     ! Decrement tally of total SUBSTRATEs
1  3420    ORIGEN$ = PLUG$
1  3430    IF SEG$(ORIGEN$, 1, 1) = "L"
2             THEN LEFT_CHAIN_OF_LEN%(CHOSEN_LENGTH%) = LEFT_CHAIN_OF_LEN%(CHOSEN_LENGTH%) - 1 &
3             \    LEFT_CHAIN_OF_LEN%(0%)             = LEFT_CHAIN_OF_LEN%(0%) - 1
1  3440    IF SEG$(ORIGEN$, 2, 2) = "R"
2             THEN RT_CHAIN_OF_LEN%(CHOSEN_LENGTH%) = RT_CHAIN_OF_LEN%(CHOSEN_LENGTH%) - 1 &
3             \    RT_CHAIN_OF_LEN%(0%)             = RT_CHAIN_OF_LEN%(0%) - 1
1  3450    CHOSEN_SITE% = FN.RAND( 1, FN.NUMB%( CHOSEN_STRING$, '1'))
1
1
1
1
1  3460!   Find Chosen site
1  3470    SEARCH,SEARCH1 = 0              ! Initialise search counters
1  3480    SEARCH = SEARCH + 1             ! Top of search loop
2  3490    IF SEG$(CHOSEN_STRING$, SEARCH, SEARCH) = '1' THEN SEARCH1 = SEARCH1 + 1  ! Count only cleav sites
2  3500    IF SEARCH1 = CHOSEN_SITE% THEN GOTO 3520   ! Exit loop on chosen site
1  3510    GOTO 3480                       ! Bottom of search loop
1
1
1
1
1
1  3520!   Counters set for cutting
1  3530    FIRST_FRAGMENT$ = SEG$(CHOSEN_STRING$, 1, SEARCH - 1)
1  3540    LENGTH_FIRST% = LEN(FIRST_FRAGMENT$) + 1
1  3550    SECOND_FRAGMENT$ = SEG$(CHOSEN_STRING$, SEARCH + 1, CHOSEN_LENGTH%)
1  3560    LENGTH_SECOND% = LEN(SECOND_FRAGMENT$) + 1
1
1
1
1
1  3570!   Test first fragment & dispose of it properly
```

```
2  3580   IF FN.CLEAVABLE(FIRST_FRAGMENT$) = 1 THEN GOTO 3630
1  3590   PRODUCTS_OF_LEN%(LENGTH_FIRST%) = PRODUCTS_OF_LEN%(LENGTH_FIRST%) + 1 ! Increment products of len
1  3600   IF SEG$(ORIGEN$, 1, 1) = "L"
2            THEN LEFT_PROD_OF_LEN%(LENGTH_FIRST%) = LEFT_PROD_OF_LEN%(LENGTH_FIRST%) + 1 &
3            \    LEFT_PROD_OF_LEN%(0%)            = LEFT_PROD_OF_LEN%(0%) + 1 &
4            \    LEFT_CHAIN_OF_LEN%(LENGTH_FIRST%) = LEFT_CHAIN_OF_LEN%(LENGTH_FIRST%) + 1 &
5            \    LEFT_CHAIN_OF_LEN%(0%)           = LEFT_CHAIN_OF_LEN%(0%) + 1
1  3610   PRODUCTS_OF_LEN%(0%) = PRODUCTS_OF_LEN%(0%) + 1 ! Increment total products
1  3620   GOTO 3750
1  3630   SUBSTRATES_OF_LEN%(LENGTH_FIRST%) = SUBSTRATES_OF_LEN%(LENGTH_FIRST%) + 1 ! Increment substrates of len
1  3640   SUBSTRATES_OF_LEN%(0%) = SUBSTRATES_OF_LEN%(0%) + 1 ! Increment total substrates
1  3650   IF SEG$(ORIGEN$, 1, 1) = "L"
2            THEN LEFT_CHAIN_OF_LEN%(LENGTH_FIRST%) = LEFT_CHAIN_OF_LEN%(LENGTH_FIRST%) + 1 &
3            \    LEFT_CHAIN_OF_LEN%(0%)           = LEFT_CHAIN_OF_LEN%(0%) + 1
1  3660   FILENAME$ = 'DRB1:[GERRY.MODELS]BASE' + STR$(LENGTH_FIRST%) + '.DAT'
1  3670   OPEN FILENAME$ AS FILE #1%, RELATIVE, ACCESS MODIFY, MAP TANK
1  3680   STORAGE$ = FIRST_FRAGMENT$
1  3690   PLUG$ = ORIGEN$
1  3700   ORIGEN$ = SEG$(ORIGEN$, 1, 1) + "X"
1  3710   PUT #1%, RECORD SUBSTRATES_OF_LEN%(LENGTH_FIRST%)
1  3720   CLOSE #1%
1
1
1
1
1  3730!  Test second fragment & dispose of it properly
1  3740   ORIGEN$ = PLUG$
2  3750   IF FN.CLEAVABLE(SECOND_FRAGMENT$) = 1 THEN GOTO 3800
1  3760   PRODUCTS_OF_LEN%(LENGTH_SECOND%) = PRODUCTS_OF_LEN%(LENGTH_SECOND%) + 1 ! Increment products of len
1  3770   IF SEG$(ORIGEN$, 2, 2) = "R"
2            THEN RT_PROD_OF_LEN%(LENGTH_SECOND%)  = RT_PROD_OF_LEN%(LENGTH_SECOND%) + 1 &
3            \    RT_PROD_OF_LEN%(0%)              = RT_PROD_OF_LEN%(0%) + 1 &
4            \    RT_CHAIN_OF_LEN%(LENGTH_SECOND%) = RT_CHAIN_OF_LEN%(LENGTH_SECOND%) + 1 &
5            \    RT_CHAIN_OF_LEN%(0%)             = RT_CHAIN_OF_LEN%(0%) + 1
1  3780   PRODUCTS_OF_LEN%(0%) = PRODUCTS_OF_LEN%(0%) + 1 ! Increment total products
1  3790   GOTO 3900
1  3800   SUBSTRATES_OF_LEN%(LENGTH_SECOND%) = SUBSTRATES_OF_LEN%(LENGTH_SECOND%) + 1 ! Increment substrates of len
1  3810   SUBSTRATES_OF_LEN%(0%) = SUBSTRATES_OF_LEN%(0%) + 1 ! Increment total substrates
1  3820   IF SEG$(ORIGEN$, 2, 2) = "R"
2            THEN RT_CHAIN_OF_LEN%(LENGTH_SECOND%) = RT_CHAIN_OF_LEN%(LENGTH_SECOND%) + 1 &
3            \    RT_CHAIN_OF_LEN%(0%)             = RT_CHAIN_OF_LEN%(0%) + 1
1  3830   FILENAME$ = 'DRB1:[GERRY.MODELS]BASE' + STR$(LENGTH_SECOND%) + '.DAT'
1  3840   OPEN FILENAME$ AS FILE #1%, RELATIVE, ACCESS MODIFY, MAP TANK
1  3850   STORAGE$ = SECOND_FRAGMENT$
1  3860   ORIGEN$ = "X" + SEG$(PLUG$, 2, 2)
1  3870   PUT #1%, RECORD SUBSTRATES_OF_LEN%(LENGTH_SECOND%)
1  3880   CLOSE #1%
1
1
1  3890!  Increment the number of cuts and check for Statistics or Finished
1
1  3900   CUTS = CUTS + 1
1
2  3910   IF ( CUTS / DO_A_COUNT_EVERY) = INT ( CUTS / DO_A_COUNT_EVERY ) THEN GOSUB 4510
2  3920   IF CUTS = TOTAL_CLEAV_SITES% THEN GOSUB 4530
2  3930   IF CUTS = TOTAL_CLEAV_SITES% THEN GOTO 5130
2
```

```
1   3940    GOTO 3050
1
1
1
1
1
1   3950!*************************************************************************************************************************
1   *****
1
1   3960!   Subroutines to correct count of random occurences for the case where the overlapping is disallowed
1
1   3970!*************************************************************************************************************************
1   ****
1
1   3980!   Determine what sequence should be added to the end of the template to cause a particular overlap.
1   3990    PRINT "OVERLAP FOUND"
1   4000    ADD_SEQ$ = SEG$(TEMPLATE$(I%),OVERLAP_LEN+1,LONG%)
2   4010    CONTENT_NO% = CONTENT_NO% + 1% \ ADD_SEQ_NO$(CONTENT_NO%)=ADD_SEQ$
1   4020    RETURN
1
1
1   4030!   Lil' Gerry's Finest Kind Combinatorics Theorem?
2   4040    LONGER$ = "" \ SHORTER$ = ""
2
1   4050!   Identify any adding sequences that are multiples of one another
1   4060    FOR V% = CONTENT_NO% TO 1 STEP -1
1   4070            FOR W% = 1 TO CONTENT_NO%
2   4080                    IF V% = W% THEN GOTO 4100
1   4090                    IF ADD_SEQ_NO$(W%) = FN.TIMES$(ADD_SEQ_NO$(V%), LEN(ADD_SEQ_NO$(W%))/LEN(ADD_SEQ_NO$(V%)))
4                               THEN SHORTER$ = ADD_SEQ_NO$(V%) \ LONGER$ = ADD_SEQ_NO$(W%) \ GOTO 4120
1   4100            NEXT W%
1   4110    NEXT V%
1
1   4120!   Keep only the shortest and longest of any set of multiple adding sequences.
2   4130    IF SHORTER$ = "" THEN SHORTER$ = "XXX"
1   4140    NON_REP_OVERLAP% = 0%
1   4150    FOR V% = 1 TO CONTENT_NO%
1   4160            IF ADD_SEQ_NO$(V%) <> FN.TIMES$(SHORTER$,LEN(ADD_SEQ_NO$(V%))/LEN(SHORTER$))
3                       THEN NON_REP_OVERLAP% = NON_REP_OVERLAP% + 1% \ TREE_SEQ$(NON_REP_OVERLAP%) = ADD_SEQ_NO$(V%)
1   4170    NEXT V%
1
1   4180!   Initial string is the template
2   4190    IF SHORTER$ = "XXX" THEN SHORTER$ = ""
2   4200    INT_TEMP$(1%) = TEMPLATE$(I%) \ INT_TEMP% = 1
1   4210    NUM_TEMP_ADDED = 0
1
1
1   4220!   Add each template to each string (provided the sum is less than the longest chain)
1   4230!   If there is a multiple sequence, the shortest of the set is added if there is an odd number of added templates,
1   4240!   and the longest of the set is added if there is an even number of added templates.
2   4250    MAT EXT_TEMP$ = NUL$ \ EXT_TEMP% = 0
1   4260    NUM_TEMP_ADDED = NUM_TEMP_ADDED + 1
1   4270    FOR W% = 1 TO INT_TEMP%
1   4280            FOR V% = 1 TO NON_REP_OVERLAP%
1   4290                    VER$ = INT_TEMP$(W%) + TREE_SEQ$(V%)
3   4300                    IF LEN(VER$) < 60 THEN EXT_TEMP% = EXT_TEMP% + 1 \ EXT_TEMP$(EXT_TEMP%) = VER$
1   4310            NEXT V%
```

```
4320           IF SHORTER$ = "" THEN GOTO 4360
4330           EXT_TEMP% = EXT_TEMP% + 1%
4340           IF NUM_TEMP_ADDED/2 = INT(NUM_TEMP_ADDED/2) THEN EXT_TEMP$(EXT_TEMP%) = INT_TEMP$(W%) + LONGER$
                                                          ELSE EXT_TEMP$(EXT_TEMP%) = INT_TEMP$(W%) + SHORTER$
4350           IF LEN(EXT_TEMP$(EXT_TEMP%)) > 60 THEN EXT_TEMP% = EXT_TEMP% - 1%
4360   NEXT W%
4370   IF EXT_TEMP% = 0 THEN GOTO 4470          ! If all the chains are too long, then the correction is made.


4380!  Pass a new set of input chains back into the above loop and
4390!  correct the expected number of random occurences
4400   MAT INT_TEMP$ = NUL$ \ INT_TEMP% = 0
4410   FOR W% = 1% TO EXT_TEMP%
4420           INT_TEMP% = INT_TEMP% + 1% \ INT_TEMP$(INT_TEMP%)=EXT_TEMP$(W%)
4430           SIGN_OF_TIMES = (-1)^NUM_TEMP_ADDED
4440           RANDOM_OCC = RANDOM_OCC + SIGN_OF_TIMES * FN.CORR(EXT_TEMP$(W%))
4450   NEXT W%

4460   GOTO 4220
4470   RETURN




4480!*****************************************************************************************************************************
***********

4490!  STATISTICS SUBROUTINE

4500!*****************************************************************************************************************************
***********


4510   ! AVERAGE MOLECULAR WEIGHT STATISTICS
4520   IF (TOTAL_CLEAV_SITES% - CUTS) < DO_A_COUNT_EVERY THEN RETURN
4530   FIRST_MOMENT, SECOND_MOMENT, THIRD_MOMENT = 0
4540   TOTAL_CHAINS_OF_LEN%(0%) = 0
4550   FOR I% = 1 TO 60
4560           TOTAL_CHAINS_OF_LEN%(I%) = SUBSTRATES_OF_LEN%(I%) + PRODUCTS_OF_LEN%(I%)
4570           FIRST_MOMENT = FIRST_MOMENT + I% * TOTAL_CHAINS_OF_LEN%(I%)
4580           SECOND_MOMENT = SECOND_MOMENT + I% * I% *  TOTAL_CHAINS_OF_LEN%(I%)
4590           THIRD_MOMENT = THIRD_MOMENT + I% * I% * I% * TOTAL_CHAINS_OF_LEN%(I%)
4600           TOTAL_CHAINS_OF_LEN%(0%) = TOTAL_CHAINS_OF_LEN%(0%) +  TOTAL_CHAINS_OF_LEN%(I%)
4610   NEXT I%
4620   NUM_AVG_MOL_WT = MOL.WT_DISACCH * FIRST_MOMENT / TOTAL_CHAINS_OF_LEN%(0%)
4630    WT_AVG_MOL_WT = MOL.WT_DISACCH * SECOND_MOMENT / FIRST_MOMENT
4640      Z_AVG_MOL_WT = MOL.WT_DISACCH * THIRD_MOMENT / SECOND_MOMENT
4650   PRINT &
       \PRINT 'After ';CUTS;' cuts,'
       \PRINT 'Number average molecular weight = ';NUM_AVG_MOL_WT
       \PRINT 'Weight average molecular weight = '; WT_AVG_MOL_WT
       \PRINT '  Z    average molecular weight = ';  Z_AVG_MOL_WT

4660!  Print the Molecular Weight Statistics out to files
4670   OPEN 'DRB1:[GERRY.MODELS]NUVSC.OUT'  AS FILE #1%, SEQUENTIAL, ACCESS APPEND
4680   PRINT #1%, CUTS;' , ';NUM_AVG_MOL_WT
```

```
1  4690    CLOSE #1%
1
1  4700    OPEN 'DRB1:[GERRY.MODELS]WTVSC.OUT'  AS FILE #1%, SEQUENTIAL, ACCESS APPEND
1  4710    PRINT #1%, CUTS;' , ';WT_AVG_MOL_WT
1  4720    CLOSE #1%
1
1  4730    OPEN 'DRB1:[GERRY.MODELS]ZVSC.OUT'  AS FILE #1%, SEQUENTIAL, ACCESS APPEND
1  4740    PRINT #1%, CUTS;' , ';Z_AVG_MOL_WT
1  4750    CLOSE #1%
1
1  4760!   Chains that can contain a chain as large as.... STATISTICS
1  4770    MAT CHAINS_AS_BIG_OR_BIGGER% = ZER
1  4780    CHAINS_AS_BIG_OR_BIGGER% (0%) = 0%
1  4790    FOR I% = 60 TO 1 STEP -1%
1  4800            CHAINS_AS_BIG_OR_BIGGER%(I%) = CHAINS_AS_BIG_OR_BIGGER%(0%) + TOTAL_CHAINS_OF_LEN%(I%)
1  4810            CHAINS_AS_BIG_OR_BIGGER%(0%) = CHAINS_AS_BIG_OR_BIGGER%(0%) + TOTAL_CHAINS_OF_LEN%(I%)
1  4820    NEXT I%
1
1
1  4830!   Name the output file for the current percentage of total degradation
1  4840    READ NOMFIL$
1  4850    DATA '00','05','10','15','20','25','30','35','40','45'
1  4860    DATA '50','55','60','65','70','75','80','85','90','95',XX
1  4870    FILE1$ = 'DRB1:[GERRY.MODELS]PROD' + NOMFIL$ + '.OUT'
1  4880    OPEN FILE1$ AS FILE #1%, SEQUENTIAL, ACCESS APPEND
1
1
1
1  4890!   Print the statistics out to a file
1  4900            PRINT #1%, CUTS;'CUTS:  ';100 * CUTS / TOTAL_CLEAV_SITES%;'% COMPLETED'
1  4910            PRINT #1%,"  CH.LEN.   TOTLCHN   CBOB      TOTLEFT   TOTLRT    TOTSUBS   TOTPROD   LEFT      RIGHT     "
1  4920    FOR I% = 0 TO 59
1  4930            PRINT #1% USING "#######", I%;
1  4940            PRINT #1%, ' , ';
1  4950            PRINT #1% USING "#######", TOTAL_CHAINS_OF_LEN%(I%);
1  4960            PRINT #1%, ' , ';
1  4970            PRINT #1% USING "#######", CHAINS_AS_BIG_OR_BIGGER%(I%);
1  4980            PRINT #1%, ' , ';
1  4990            PRINT #1% USING "#######", LEFT_CHAIN_OF_LEN%(I%);
1  5000            PRINT #1%, ' , ';
1  5010            PRINT #1% USING "#######", RT_CHAIN_OF_LEN%(I%);
1  5020            PRINT #1%, ' , ';
1  5030            PRINT #1% USING "#######", SUBSTRATES_OF_LEN%(I%);
1  5040            PRINT #1%, ' , ';
1  5050            PRINT #1% USING "#######", PRODUCTS_OF_LEN%(I%);
1  5060            PRINT #1%, ' , ';
1  5070            PRINT #1% USING "#######", LEFT_PROD_OF_LEN%(I%);
1  5080            PRINT #1%, ' , ';
1  5090            PRINT #1% USING "#######", RT_PROD_OF_LEN%(I%)
1  5100    NEXT I%
1  5110    CLOSE 1%
1  5120    RETURN
1
1  5130    !EXIT FROM THE PROGRAM
1  5140    END
```

VARIABLES

Variables for COM/MAP - TANK

Name	Type	Offset		Size (Bytes)	Dim 1	Dim 2
STORAGE$	STRING	0	00000000			
ORIGEN$	STRING	60	0000003C			
Total space		62	0000003E			

Numeric scalar variables

Name	Type	Offset(R11)	
JUICE	SINGLE	123	0000007B
CHANCES_OF_CHOOSING_LEN	SINGLE	119	00000077
A	SINGLE	115	00000073
POSSIBLE	SINGLE	111	0000006F
OVERALL_UNCLEAV	SINGLE	107	0000006B
B	SINGLE	103	00000067
C	SINGLE	99	00000063
NUMBER_CLEAV	SINGLE	95	0000005F
PROB_FREE_0	SINGLE	91	0000005B
COIN_FLIP	SINGLE	87	00000057
DESIRED_OCC	SINGLE	83	00000053
PROB_FREE_1	SINGLE	79	0000004F
COUNT_OVER	SINGLE	75	0000004B
COINFLIP	SINGLE	71	00000047
RANDOM_OCC	SINGLE	67	00000043
DO_A_COUNT_EVERY	SINGLE	63	0000003F
SUBSTRATE_LEN_SEL_INDEX	SINGLE	59	0000003B
NUMBER_UNCLEAV	SINGLE	55	00000037
TEMPLATE_WT	SINGLE	51	00000033
SEARCH	SINGLE	47	0000002F
NUM_TEMP_ADDED	SINGLE	43	0000002B
SIGN_OF_TIMES	SINGLE	39	00000027
OVERLAP_LEN	SINGLE	35	00000023
PROB_OF_MUTATION_TO_LEN	SINGLE	31	0000001F
SEARCH1	SINGLE	27	0000001B
FIRST_MOMENT	SINGLE	23	00000017
Z_AVG_MOL_WT	SINGLE	19	00000013
WT_AVG_MOL_WT	SINGLE	15	0000000F
NUMBER_FREE	SINGLE	11	0000000B
OUT_TO	SINGLE	7	00000007
THIRD_MOMENT	SINGLE	3	00000003
MUTATION_SEL_INDEX	SINGLE	-1	FFFFFFFF
K	SINGLE	-5	FFFFFFFB
SECOND_MOMENT	SINGLE	-9	FFFFFFF7
L	SINGLE	-13	FFFFFFF3
NUM_AVG_MOL_WT	SINGLE	-17	FFFFFFEF
OVERALL_CLEAV	SINGLE	-21	FFFFFFEB
MOL.WT_DISACCH	SINGLE	-25	FFFFFFE7
OVERLAP_TRAP	SINGLE	-29	FFFFFFE3

CUTS	SINGLE	-33	FFFFFFDF
K%	LONG	-37	FFFFFFDB
L%	LONG	-41	FFFFFFD7
CHOSEN_SUBSTRATE%	LONG	-45	FFFFFFD3
LENGTH_SECOND%	LONG	-49	FFFFFFCF
LONG%	LONG	-53	FFFFFFCB
INT_TEMP%	LONG	-57	FFFFFFC7
A%	LONG	-61	FFFFFFC3
CHOSEN_LENGTH%	LONG	-65	FFFFFFBF
TO%	LONG	-69	FFFFFFBB
T%	LONG	-73	FFFFFFB7
CHOSEN_SITE%	LONG	-77	FFFFFFB3
TOTAL_CLEAV_SITES%	LONG	-81	FFFFFFAF
SKIP_P%	LONG	-85	FFFFFFAB
V%	LONG	-89	FFFFFFA7
EXT_TEMP%	LONG	-93	FFFFFFA3
CONTENT_NO%	LONG	-97	FFFFFF9F
W%	LONG	-101	FFFFFF9B
I%	LONG	-105	FFFFFF97
LENGTH_FIRST%	LONG	-109	FFFFFF93
NON_REP_OVERLAP%	LONG	-113	FFFFFF8F
CHAINS_AS_BIG_OR_BIGGER%	LONG	-117	FFFFFF8B
J%	LONG	-121	FFFFFF87

User functions arguments and locals

Name	Type	Offset(R10)	
FN.RAND	SINGLE	123	0000007B
A	SINGLE	119	00000077
B	SINGLE	115	00000073
FN.NUMB%	LONG	123	0000007B
A$	STRING	119	00000077
B$	STRING	115	00000073
FN.CLEAVABLE	SINGLE	123	0000007B
A$	STRING	119	00000077
FN.CORR	SINGLE	123	0000007B
A$	STRING	119	00000077
A$	STRING	123	0000007B
A	SINGLE	119	00000077
FN.TIMES$	STRING	115	00000073
FN.MAX	SINGLE	123	0000007B
A	SINGLE	119	00000077
B	SINGLE	115	00000073
FN.MIN	SINGLE	123	0000007B
A	SINGLE	119	00000077
B	SINGLE	115	00000073

DESCRIPTORS

Dynamic array descriptors

Name	Type	Offset(R11)	
MUTATION_SEL_INDEX	SINGLE	-165	FFFFFF5B
PROB_OF_MUTATION_TO_LEN	SINGLE	-209	FFFFFF2F
EXT_TEMP$	STRING	-241	FFFFFF0F
CHAINS_AS_BIG_OR_BIGGER%	LONG	-273	FFFFFEEF
POSSIBLE	SINGLE	-317	FFFFFEC3
ADD_SEQ_NO$	STRING	-349	FFFFFEA3
SUBSTRATE_LEN_SEL_INDEX	SINGLE	-381	FFFFFE83
INT_TEMP$	STRING	-413	FFFFFE63
CHANCES_OF_CHOOSING_LEN	SINGLE	-445	FFFFFE43
COUNT_OVER	SINGLE	-477	FFFFFE23

Dynamic string scalar descriptors

Name	Type	Offset(R11)	
YODA$	STRING	-485	FFFFFE1B
ADD_SEQ_NO$	STRING	-493	FFFFFE13
VER$	STRING	-501	FFFFFE0B
FIRST_PART$	STRING	-509	FFFFFE03
SECOND_FRAGMENT$	STRING	-517	FFFFFDFB
PLACE_OVER$	STRING	-525	FFFFFDF3
OVER_PLACE$	STRING	-533	FFFFFDEB
INT_TEMP$	STRING	-541	FFFFFDE3
FILENAME$	STRING	-549	FFFFFDDB
ADD_SEQ$	STRING	-557	FFFFFDD3
FILE1$	STRING	-565	FFFFFDCB
B$	STRING	-573	FFFFFDC3
SECOND_PART$	STRING	-581	FFFFFDBB
CHAIN$	STRING	-589	FFFFFDB3
CHOSEN_PLACE$	STRING	-597	FFFFFDAB
NOMFIL$	STRING	-605	FFFFFDA3
CHOSEN_STRING$	STRING	-613	FFFFFD9B
EXT_TEMP$	STRING	-621	FFFFFD93
SKIP$	STRING	-629	FFFFFD8B
LONGER$	STRING	-637	FFFFFD83
SHORTER$	STRING	-645	FFFFFD7B
IN1$	STRING	-653	FFFFFD73
OUT$	STRING	-661	FFFFFD6B
PLUG$	STRING	-669	FFFFFD63
IN2$	STRING	-677	FFFFFD5B
FIRST_FRAGMENT$	STRING	-685	FFFFFD53

Dynamic string array element descriptors

Name	Type	Offset(R11)		Size (Bytes)		Dim 1	Dim 2
TEMPLATE$	STRING	-733	FFFFFD23	48	00000030	5	0
TREE_SEQ$	STRING	-1221	FFFFFB3B	488	000001E8	60	0

INT_TEMP$	STRING	-2269	FFFFF723	1048	00000418	130	0
ADD_SEQ_NO$	STRING	-2757	FFFFF53B	488	000001E8	60	0
EXT_TEMP$	STRING	-7965	FFFFE0E3	5208	00001458	650	0

Dynamic numeric array elements

Name	Type	Offset(R11)		Size (Bytes)		Dim 1	Dim 2
LEFT_CHAIN_OF_LEN%	LONG	-8209	FFFFDFEF	244	000000F4	60	0
COUNT_OVER	SINGLE	-8233	FFFFDFD7	24	00000018	5	0
CHANCES_OF_CHOOSING_LEN	SINGLE	-8477	FFFFDEE3	244	000000F4	60	0
TOTAL_CHAINS_OF_LEN%	LONG	-8721	FFFFDDEF	244	000000F4	60	0
RANDOM_OCC_TEMPLATE	SINGLE	-8765	FFFFDDC3	44	0000002C	10	0
SKIPPY	SINGLE	-8809	FFFFDD97	44	0000002C	10	0
ABS_AFFIN_FOR_SUBSTRATE_LEN	SINGLE	-9053	FFFFDCA3	244	000000F4	60	0
JUICE_PROB_TEMPLATE	SINGLE	-9077	FFFFDC8B	24	00000018	5	0
SUBSTRATE_LEN_SEL_INDEX	SINGLE	-9321	FFFFDB97	244	000000F4	60	0
RT_PROD_OF_LEN%	LONG	-9565	FFFFDAA3	244	000000F4	60	0
SKIPPY%	LONG	-9589	FFFFDA8B	24	00000018	5	0
POSSIBLE	SINGLE	-11053	FFFFD4D3	1464	000005B8	5	60
TEMPLATE_NUMBER%	LONG	-11097	FFFFD4A7	44	0000002C	10	0
REL_AFFIN_FOR_SUBSTRATE_LEN	SINGLE	-11341	FFFFD3B3	244	000000F4	60	0
CHAINS_AS_BIG_OR_BIGGER%	LONG	-11585	FFFFD2BF	244	000000F4	60	0
LEFT_PROD_OF_LEN%	LONG	-11829	FFFFD1CB	244	000000F4	60	0
SUBSTRATES_OF_LEN%	LONG	-12073	FFFFD0D7	244	000000F4	60	0
MERS_IN_SUBSTRATES_OF_LEN%	LONG	-12317	FFFFCFE3	244	000000F4	60	0
PRODUCTS_OF_LEN%	LONG	-12561	FFFFCEEF	244	000000F4	60	0
RT_CHAIN_OF_LEN%	LONG	-12805	FFFFCDFB	244	000000F4	60	0
PROB_OF_MUTATION_TO_LEN	SINGLE	-14269	FFFFC843	1464	000005B8	5	60
MUTATION_SEL_INDEX	SINGLE	-15733	FFFFC28B	1464	000005B8	5	60
DESIRED_OCC_TEMPLATE	SINGLE	-15777	FFFFC25F	44	0000002C	10	0
JUICE_OCC_TEMPLATE	SINGLE	-15821	FFFFC233	44	0000002C	10	0

TEMPORARIES

Name	Type	Offset(R9)	
.TMP 1	STRING	0	00000000
.TMP 2	STRING	8	00000008
.TMP 3	STRING	16	00000010
.TMP 4	STRING	24	00000018
.TMP 17	STRING	-8	FFFFFFF8
.TMP 18	STRING	-16	FFFFFFF0
.TMP 19	STRING	-24	FFFFFFE8
.TMP 33	LONG	-28	FFFFFFE4
.TMP 34	LONG	-32	FFFFFFE0
.TMP 36	LONG	-36	FFFFFFDC
.TMP 38	LONG	-40	FFFFFFD8
.TMP 39	LONG	-44	FFFFFFD4
.TMP 40	LONG	-48	FFFFFFD0
.TMP 41	LONG	-52	FFFFFFCC
.TMP 42	LONG	-56	FFFFFFC8
.TMP 35	SINGLE	-60	FFFFFFC4
.TMP 37	SINGLE	-64	FFFFFFC0

Final dynamic offset	-15917	FFFFC1D3
Total variable space	16044	00003EAC
Stack space in pages	32	00000020

PROGRAM SECTIONS

	Name	Bytes	Attributes
0	$PDATA	4624	PIC CON REL LCL SHR NOEXE RD NOWRT LONG
1	$CODE	13449	PIC CON REL LCL SHR EXE RD NOWRT LONG
2	TANK	62	PIC OVR REL GBL SHR NOEXE RD WRT LONG

EXTERNAL REFERENCES

OTS$LINKAGE	BAS$LINKAGE	BAS$POWRJ	BAS$POWRR
STR$TRIM	STR$CONCAT	BAS$SEG	BAS$STR_L
BAS$VAL_F	BAS$INIT_R8	BAS$INIT_DEF_R8	BAS$INIT_GOSUB
BAS$END_R8	BAS$END_DEF_R8	BAS$END_GSB_R8	BAS$INPUT
BAS$READ	BAS$PRINT	BAS$PRINT_USING	BAS$IO_END
BAS$IN_L_R	BAS$IN_F_R	BAS$IN_T_DX	BAS$OUT_L_V_S
BAS$OUT_L_V_B	BAS$OUT_F_V_S	BAS$OUT_F_V_B	BAS$OUT_T_DX_S
BAS$OUT_T_DX_B	BAS$OUT_T_DX_C	BAS$OPEN	BAS$CLOSE
BAS$GET_RECORD	BAS$PUT	BAS$PUT_RECORD	BAS$DELETE
BAS$UPDATE	BAS$STOP	BAS$MAT_NULL	BAS$MAT_INIT
MTH$FLOOR_R1	BAS$RND_F_R1	BAS$RANDOMIZE	STR$COPY_R_R8
STR$COPY_DX_R8			

LINE NUMBER CROSS-REFERENCE TABLE

Line	References		
670	630.002		
1120	1520.001		
1190	1160.002		
1260	1310.003		
1450	1320.002		
1490	1540.003		
1530	1470.002		
1550	1460.002		
1620	1170.002		
1840	1980.001		
1970	1920.002	1930.003	1940.002
1990	1840.002		
2140	2040.002		
2160	2130.001		
2450	2390.002		
2550	2500.002		
2610	2670.002	2840.002	
2850	2800.002	2830.002	
2940	2580.002	2590.002	
3050	3940.001		
3200	3250.002		
3400	3350.002		
3480	3510.001		
3520	3500.002		
3630	3580.002		

3750	3620.001		
3800	3750.002		
3900	3790.001		
3980	1410.003		
4030	1440.002		
4100	4080.002		
4120	4090.004		
4220	4460.001		
4360	4320.002		
4470	4370.002		
4510	2240.001	3910.002	
4530	3130.003	3920.002	5140.001
5130	3130.004	3930.002	

SYMBOL CROSS-REFERENCE TABLE

Symbol	Class	References							
A	L	610.001#	630.001	640.001					
A	L	690.001#	700.001	700.002					
A	L	320.001#	340.001	350.001	360.001				
A	L	720.001#	730.001	730.002					
A		460.001	480.001	510.001					
A$	L	530.001#	560.001	570.001	590.001				
A$	L	380.001#	400.001	410.001					
A$	L	610.001#	650.001						
A$	L	450.001#	470.001	480.001	490.001				
A%		560.001	570.001	580.001					
ABS_AFFIN_FOR_SUBSTRATE_LEN	I	210.001#	3160.001	3170.001					
ADD_SEQ$		4000.001	4010.002						
ADD_SEQ_NO$	I	250.001#	1190.001	4010.002	4090.001	4090.002	4090.003	4160.001	4160.003

YODA$	1130.001	1140.001	1140.002	1150.001	1160.001	1170.001	1180.001
Z_AVG_MOL_WT	4640.001	4650.008	4740.001				

Key for special characters above:

```
+-------------------------------------+
!                                     !
!   C = Constant                      !
!   E = External                      !
!   F = user defined Function         !
!   I = subscripted ( Indexed )       !
!   L = Local parameter to function   !
!   M = Map or common name            !
!   S = Subprogram name               !
!   V = Virtual array                 !
!                                     !
!   # = explicit definition           !
!                                     !
+-------------------------------------+
```

Compilation qualifiers in effect

```
/CHECK=(BOUNDS,OVERFLOW)
/DEBUG=(NOSYMBOLS,TRACEBACK)
/LONG  /SINGLE  /LINES  /SETUP
/OBJECT  /LISTING  /NOMACHINE  /CROSS
```

APPENDIX B

Computer Program for Calculations Assuming the External Reaction Model

This appendix contains a short BASIC program that was used to predict the rate of reaction of immobilized heparinase using the assumptions of the external transport model. This program is very useful for ascertaining the effects of varying parameters singly or together. All of the parameters used to estimate the rate of reaction were obtained from either the manufacturer or from experiments performed in this laboratory. The model system is the *in vivo* system described in Langer et al.[113] Lines 10 to 30 of the program are the assumed constants for blood in large vessels at 30C. Lines 40-42 contain the radius of Sepharose 4B beads reported by Pharmacia[114] and the corresponding volume and external surface area of a single bead. The Michaelis constants for the free enzyme are taken from Flanagan.[115] Lines 70 to 77 contain the specifications given for the model reactor. The velocity of the fluid in the bed was kept at 300 ml/min by a recirculation pump. The bed volume of the Sepharose beads was 50 ml. A total of 5.5 mg of protein was immobilized to the beads. The enzyme was hydroxylapatite purified,[116] but the exact concentration of pure enzyme is unknown. The cross-sectional area of the reactor is computed in line 73, assuming an average reactor diameter of 6

---

113. Langer, R., R.J. Linhardt, S. Hoffberg, A.K. Larsen, C.L. Cooney, D. Tapper, and M. Klein, "An Enzymatic System for Removing Heparin in Extracorporeal Therapy," *Science*, 217, 261-263 (1982).

114. Pharmacia Fine Chemicals Company, *Affinity Chromatography: Principles and Methods*. Pharmacia Fine Chemicals, Uppsala, Sweden.

115. Flanagan, M.M., "Purification and Characterization of Heparinase," S.M. Thesis, MIT, Sept., 1981.

116. *ibid*.

cm. The concentration of heparin at the input to the reactor is taken as 4 units per ml of blood, an average therapeutic dose. The number of beads in the reactor and the total external surface area of the beads is computed based on face-centered cubic packing of the beads when the volume is taken. The beads pack loosely, and face-centered cubic packing is the loosest form of packing for spheres. The activity of the heparin is taken as 160 units per mg, as specified by the Sigma Chemical Company on the label of their heparin. Average molecular weights were taken as 12,800 daltons for heparin and 50,000 daltons for heparinase. The turnover number of the enzyme, k_{cat}, was computed from the reported value of V_m and the amount of enzyme used in the assay (assuming that the HA purified heparinase was pure).[117] The variable VMI is the turnover number per unit external area of bead. This variable contains a factor of .9 to take into account the 10% loss of activity upon immobilization.

After the variables normally measured in non-MKS units are converted to MKS units, the rate of heparin transport from bulk solution to the surface of the bead and the rate of reaction of heparin at the surface of the bead are computed as described in the THEORY section. The concentration of heparin at the surface of the bead (the variable CSOU) is varied until the two rates are equal. This rate is taken as the rate of the reaction.

---

117. ibid.

```
1   1     PIE   = 3.141593                                  !--              Numeric constant
1   10    DIFF = 1E-10                                      !M^2 S^-1        Water property
1   20    VISC = .7975E-3                                   !KG M^-1 S^-1          "
1   30    DENS = 1000                                       !KG M^-3               "
1
1   40    RADI = 60E-6                                      !M               Bead property
1   41    AREA = 4 * PIE * RADI^2                           !M^2             Surface area of bead
1   42    VOLB = 4 * PIE * RADI^3 / 3                       !M^3             Volume of bead
1
1
1   50    VM   = 4.24E-4 / 3600                             !MOL L^-1 S^-1   Enzyme constant
1   60    KM   = 3.16E-5 * 1000                             !MOL M^3               "
1
1
1   70    VFLU = 300                                        !ML MIN^-1       Flow rate inside reactor
1   71    VOLUME_BED = 50                                   !ML              Bed Volume
1   72    ENZYME_IMM = 5.5                                  !MG              Enzyme immobilized
1   73    AREA_RXTOR = PIE * .03^2                          !M^2             Cross-sectional area of reactor
1   75    CSBL = 4                                          !UNITS ML^-1     Initial concentration of hepain in blood
1   76    NUM_BEADS = (VOLUME_BED / 1E6) / (8*RADI^3)       !--              Number of beads in suspension
1   77    TOTL_AREA = NUM_BEADS * AREA                      !M^2             Total bead area
1
1
1   80    ACTIV = 160                                       !UNITS MG^-1     Heparin activity
1   85    MWHEP = 12800                                     !G MOL^-1        Heparin molecular weight
1   90    MWENZ = 50000                                     !G MOL^-1        Enzyme molecular weight
1
1   100   KCAT  = VM * MWENZ * 6E-4 / 5E-6                  !--              Turnover number
1   110   VMI   = KCAT * (ENZYME_IMM / (1000 * MWENZ)) / TOTL_AREA
1   111                                                     !MOL M^-2 S^-1   Area based maximum velocity
1   120   VMI   = VMI * .9                                  !MOL M^-2 S^-1   Loss of activity on immobilization
1
1   180!  Convert above numbers to MKS
1   190   VINF = VFLU / 60E6                                !M^3 S^-1        Velocity of bulk solution
1   195   VINF = VINF / AREA_RXTOR                          !M S^-1          Linear velocity of bulk solution
1   200   CINF = CSBL * 1000 / (ACTIV * MWHEP)              !MOL M^-3        Concentration in bulk solution
1   210   PRINT "Concentration at infinity";CINF
1
1
1   250   INPUT"CS",CSOU                                    !MOL M^-3        Heparin conc. @ surface
1
1
1   400!  Compute intermediate values
1
1   401!  External Diffusion      (Assuming spherical Mass Transfer Coeff.)
1   420   RE   = 2 * RADI * VINF * DENS / VISC              !--              Reynolds number
1   430   SC   = VISC / (DENS * DIFF)                       !--              Schmitt number
1   440   BETA = (DIFF / (2*RADI)) * (2 + .6 * RE^.5 * SC^(1/3))
1   441                                                     !M S^-1          Mass transfer coefficent
1   460   VEXT = BETA * (CINF - CSOU)                       !MOL M^-2 S^-1   Mass transfer per unit area
1   465   VEX  = VEXT * TOTL_AREA                           !MOL S^-1        Total rate of mass transfer
1   470   PRINT "Velocity of external mass transport = ";VEX;" MOL / S"
1
1   500   !Reaction
1   510   VNT = VMI * CSOU  / KM                            !MOL M^2 S^-1    Reaction per unit area
1   520   VINT = VNT * TOTL_AREA                            !MOL S^-1        Total rate of reaction
1
```

```
    1   650     PRINT "Velocity of reaction = ";VINT;" MOL / S"
    1
    1
    3   800     IF VINT = VEX THEN GOTO 1000 ELSE GOTO 250
    3
    1   1000    END
    1
    1
```

VARIABLES

Numeric scalar variables

Name	Type	Offset(R11)	
RADI	DOUBLE	119	00000077
CINF	DOUBLE	111	0000006F
MWHEP	DOUBLE	103	00000067
MWENZ	DOUBLE	95	0000005F
VINT	DOUBLE	87	00000057
VOLUME_BED	DOUBLE	79	0000004F
VOLB	DOUBLE	71	00000047
VM	DOUBLE	63	0000003F
KCAT	DOUBLE	55	00000037
VINF	DOUBLE	47	0000002F
VEX	DOUBLE	39	00000027
CSBL	DOUBLE	31	0000001F
VISC	DOUBLE	23	00000017
SC	DOUBLE	15	0000000F
AREA_RXTOR	DOUBLE	7	00000007
ACTIV	DOUBLE	-1	FFFFFFFF
RE	DOUBLE	-9	FFFFFFF7
VEXT	DOUBLE	-17	FFFFFFEF
KM	DOUBLE	-25	FFFFFFE7
VNT	DOUBLE	-33	FFFFFFDF
DIFF	DOUBLE	-41	FFFFFFD7
AREA	DOUBLE	-49	FFFFFFCF
DENS	DOUBLE	-57	FFFFFFC7
ENZYME_IMM	DOUBLE	-65	FFFFFFBF
CSOU	DOUBLE	-73	FFFFFFB7
TOTL_AREA	DOUBLE	-81	FFFFFFAF
VMI	DOUBLE	-89	FFFFFFA7
BETA	DOUBLE	-97	FFFFFF9F
VFLU	DOUBLE	-105	FFFFFF97
PIE	DOUBLE	-113	FFFFFF8F
NUM_BEADS	DOUBLE	-121	FFFFFF87
Final dynamic offset		-121	FFFFFF87
Total variable space		248	000000F8
Stack space in pages		1	00000001

PROGRAM SECTIONS

	Name	Bytes	Attributes
0	$PDATA	347	PIC CON REL LCL SHR NOEXE RD NOWRT LONG
1	$CODE	974	PIC CON REL LCL SHR EXE RD NOWRT LONG

EXTERNAL REFERENCES

OTS$LINKAGE	BAS$LINKAGE	BAS$POWDD	BAS$INIT_R8
BAS$END_R8	BAS$INPUT	BAS$PRINT	BAS$IO_END
BAS$IN_D_R	BAS$OUT_D_V_S	BAS$OUT_D_V_B	BAS$OUT_T_DX_S
BAS$OUT_T_DX_B	BAS$OUT_T_DX_C		

LINE NUMBER CROSS-REFERENCE TABLE

Line	References
250	800.003
1000	800.003

SYMBOL CROSS-REFERENCE TABLE

Symbol	Class	References					
ACTIV		80.001	200.001				
AREA		41.001	77.001				
AREA_RXTOR		73.001	195.001				
BETA		440.001	460.001				
CINF		200.001	210.001	460.001			
CSBL		75.001	200.001				
CSOU		250.001	460.001	510.001			
DENS		30.001	420.001	430.001			
DIFF		10.001	430.001	440.001			
ENZYME_IMM		72.001	110.001				
KCAT		100.001	110.001				
KM		60.001	510.001				
MWENZ		90.001	100.001	110.001			
MWHEP		85.001	200.001				
NUM_BEADS		76.001	77.001				
PIE		1.001	41.001	42.001	73.001		
RADI		40.001	41.001	42.001	76.001	420.001	440.001
RE		420.001	440.001				
SC		430.001	440.001				
TOTL_AREA		77.001	110.001	465.001	520.001		

VEX	465.001	470.001	800.001
VEXT	460.001	465.001	
VFLU	70.001	190.001	
VINF	190.001	195.001	420.001
VINT	520.001	650.001	800.001
VISC	20.001	420.001	430.001
VM	50.001	100.001	
VMI	110.001	120.001	510.001
VNT	510.001	520.001	
VOLB	42.001		
VOLUME_BED	71.001	76.001	

Key for special characters above:

```
+-----------------------------------+
!                                   !
!   C = Constant                    !
!   E = External                    !
!   F = user defined Function       !
!   I = subscripted ( Indexed )     !
!   L = Local parameter to function !
!   M = Map or common name          !
!   S = Subprogram name             !
!   V = Virtual array               !
!                                   !
!   # = explicit definition         !
!                                   !
+-----------------------------------+
```

Compilation qualifiers in effect

```
/CHECK=(BOUNDS,OVERFLOW)
/DEBUG=(NOSYMBOLS,TRACEBACK)
/LONG  /DOUBLE  /SCALE=0  /LINES  /SETUP
/OBJECT  /LISTING  /NOMACHINE  /CROSS
```

APPENDIX C

Computer Program for Calculations Assuming the Internal Reaction Model

This appendix contains a short BASIC program that was used to predict the rate of reaction of immobilized heparinase using the assumptions of the internal transport model. This program is very useful for ascertaining the effects of varying parameters singly or together. All of the parameters used to estimate the rate of reaction were obtained from either the manufacturer or from experiments performed in this laboratory. The model system is the *in vivo* system described in Langer et al.[118] Lines 10 to 30 of the program are the assumed constants for blood in large vessels at 30C. Lines 40-42 contain the radius of Sepharose 4B beads reported by Pharmacia[119] and the corresponding volume and external surface area of a single bead. The electrostatic potential of the bead is taken as -48 millivolts (see THEORY section). The tortuosity of Sepharose beads is given as 3.[120] The diffusion constant of heparin inside the bead (ignoring electrostatic effects) is computed as the diffusion constant of heparin in blood divided by the tortuosity of the bead. The Michaelis constants for the free enzyme are taken from Flanagan.[121] Lines 70 to 77 contain the specifications given for the model reactor. The velocity of the fluid in the bed was kept at 300 ml/min by

---

118. Langer, R., Linhardt, R.J., Hoffberg, S., Larsen, A.K., Cooney, C.L., Tapper, D., and Klein, M., "An Enzymatic System for Removing Heparin in Extracorporeal Therapy," Science, 217, 261-263 (1982).

119. Pharmacia Fine Chemicals Company, Affinity Chromatography: Principles and Methods. Pharmacia Fine Chemicals, Uppsala, Sweden.

120. ibid.

121. Flanagan, M.M., "Purification and Characterization of Heparinase," S.M. Thesis, MIT, Sept., 1981.

a recirculation pump. The bed volume of the Sepharose beads was 50 ml. A total of 5.5 mg of protein was immobilized to the beads. The enzyme was hydroxylapatite purified,[122] but the exact concentration of pure enzyme is unknown. The cross-sectional area of the reactor is computed in line 73, assuming an average reactor diameter of 6 cm. The concentration of heparin at the input to the reactor is taken as 4 units per ml of blood, an average therapeutic dose. The number of beads in the reactor and the total external surface area of the beads is computed based on face-centered cubic packing of the beads when the volume is taken. The beads pack loosely, and face-centered cubic packing is the loosest form of packing for spheres. The activity of the heparin is taken as 160 units per mg, as specified by the Sigma Chemical Company on the label of their heparin. Average molecular weights were taken as 12,800 daltons for heparin and 50,000 daltons for heparinase. The charge on the heparin molecule is contained in the variable Z, given here as -55.4, but many values of Z were used in this study. The turnover number of the enzyme, k_{cat}, was computed from the reported value of V_m and the amount of enzyme used in the assay (assuming that the HA purified heparinase was pure).[123] The variable VMI is the turnover number per unit volume of bead. This variable contains a factor of .9 to take into account the 10% loss of activity upon immobilization.

After the variables normally measured in non-MKS units are converted to MKS units, the rate of heparin transport from bulk solution to the surface of the bead and the rate of reaction of heparin inside the bead are computed as

---

122. ibid.

123. ibid.

described in the THEORY section. The concentration of heparin at the surface of the bead (the variable CSOU) is varied until the two rates are equal. This rate is taken as the rate of the reaction.

```
1   1     PIE   = 3.141593                                !--           Numeric constant
1   2     T     = 303                                     !K            Temperature of reaction
1   4     R     = 1.987                                   !CAL K^-1     Gas constant
1   5     FARA  = 23063                                   !CAL EQ^-1 V^-1 Faraday's constant
1
1   10    DIFF = 1E-10                                    !M^2 S^-1     Water property
1   20    VISC = .7975E-3                                 !KG M^-1 S^-1       "
1   30    DENS = 1000                                     !KG M^-3            "
1
1   40    RADI = 60E-6                                    !M            Bead radius
1   41    AREA = 4 * PIE * RADI^2                         !M^2          Surface area of bead
1   42    VOLB = 4 * PIE * RADI^3 / 3                     !M^3          Volume of bead
1   45    PSI  = -.048                                    !V            ES Potential of bead
1   46    TORT = 3                                        !--           Tortuosity of bead
1   47    DIFFE = DIFF / TORT                             !M^2 S^-1     Effective diffusivity in pores
1
1
1   50    VM   = 4.24E-4 / 3600                           !MOL L^-1 S^-1  Enzyme constant
1   60    KM   = 3.16E-5 * 1000                           !MOL M^-3           "
1
1
1   70    VFLU = 300                                      !ML MIN^-1    Flow rate inside reactor
1   71    VOLUME_BED = 50                                 !ML           Bed Volume
1   72    ENZYME_IMM = 5.5                                !MG           Enzyme immobilized
1   73    AREA_RXTOR = PIE * .03^2                        !M^2          Cross-sectional area of reactor
1   75    CSBL = 4                                        !UNITS ML^-1  Initial concentration of hepain in blood
1   76    NUM_BEADS = (VOLUME_BED / 1E6) / VOLB           !--           Number of beads in suspension
1   77    TOTL_AREA = NUM_BEADS * AREA                    !M^2          Total bead area
1   78    TOTL_VOLU = NUM_BEADS * VOLB                    !M^3          Total bead volume
1
1
1   80    ACTIV = 160                                     !UNITS MG^-1  Heparin activity
1   85    MWHEP = 12800                                   !G MOL^-1     Heparin molecular weight
1   86    Z     = -55.4                                   !EQ MOL^-1    Avg. charge on heparin
1   90    MWENZ = 50000                                   !G MOL^-1     Enzyme molecular weight
1
1   100   KCAT  = VM * MWENZ * 6E-4 / 5E-6                !--           Turnover number
1   110   VMI   = KCAT * (ENZYME_IMM / (1000 * MWENZ)) / TOTL_VOLU
1   111                                                   !MOL M^-3 S^-1  Volume based maximum velocity
1   120   VMI   = VMI * .9                                !MOL M^-3 S^-1  Loss of activity on immobilization
1
1   180!  Convert above numbers to MKS
1   190   VINF = VFLU / 60E6                              !M^3 S^-1     Velocity of bulk solution
1   195   VINF = VINF / AREA_RXTOR                        !M S^-1       Linear velocity of bulk solution
1   200   CINF = CSBL * 1000 / (ACTIV * MWHEP)            !MOL M^-3     Concentration in bulk solution
1   210   PRINT "Concentration at infinity";CINF
1
1
1   250   INPUT"CS",CSOU                                  !MOL M^-3     Heparin conc. @ surface
1
1
1   400!  Compute intermediate values
1
1   401!  External Diffusion      (Assuming spherical Mass Transfer Coeff.)
1   420   RE   = 2 * RADI * VINF * DENS / VISC            !--           Reynolds number
1   430   SC   = VISC / (DENS * DIFF)                     !--           Schmitt number
1   440   BETA = (DIFF / (2*RADI)) * (2 + .6 * RE^.5 * SC^(1/3))
```

```
1   441                                                     !M S^-1          Mass transfer coefficent
1   460     VEXT = BETA * (CINF - CSOU)                     !MOL M^-2 S^-1   Mass transfer per unit area
1   465     VEX  = VEXT * TOTL_AREA                         !MOL S^-1        Total rate of mass transfer
1   470     PRINT "Velocity of external mass transport = ";VEX;" MOL / S"
1
1   490!    Define hyperbolic tangent
1
1   500     DEF FN.TANH(X)
3   501             IF X > 80 THEN FN.TANH = 1 ELSE FN.TANH = (EXP(X) - EXP(-X)) / (EXP(X) + EXP(-X))
1   502     FNEND
1
1   509!    Partition
1   510     LAMBDA = Z * FARA * PSI / (R * T)               !--              Partition coefficient
1   520     CSIN = CSOU * EXP(-LAMBDA/2.303)                !MOL M^-3        Internal surface conc.
1
1   601!    Internal transfer & reaction
1   610     THEILE = RADI * (VM  / (KM * DIFFE))^.5 / 3     !--              Theile modulus
1   620     ETA = THEILE^-1 * ( 1 / FN.TANH( 3 * THEILE) - 1 / (3*THEILE))
1   621                                                     !--              Effectiveness factor
1   622     PRINT "ETA",ETA
1   630     VSP = VM * CSIN / KM                            !MOL M^-3 S^-1   Reaction rate at surface per bead
1   640     VINT = ETA * VSP * TOTL_VOLU                    !MOL S^-1        Total reaction rate
1
1
1   650     PRINT "Velocity of reaction = ";VINT;" MOL / S"
1
1
3   800     IF VINT = VEX THEN GOTO 1000 ELSE GOTO 250
3
1   1000    END
1
1
```

VARIABLES

Numeric scalar variables

Name	Type	Offset(R11)	
RADI	DOUBLE	119	00000077
CINF	DOUBLE	111	0000006F
MWHEP	DOUBLE	103	00000067
MWENZ	DOUBLE	95	0000005F
LAMBDA	DOUBLE	87	00000057
VINT	DOUBLE	79	0000004F
R	DOUBLE	71	00000047
VOLUME_BED	DOUBLE	63	0000003F
VOLB	DOUBLE	55	00000037
VM	DOUBLE	47	0000002F
KCAT	DOUBLE	39	00000027
VINF	DOUBLE	31	0000001F
VEX	DOUBLE	23	00000017
T	DOUBLE	15	0000000F
CSBL	DOUBLE	7	00000007
VISC	DOUBLE	-1	FFFFFFFF
SC	DOUBLE	-9	FFFFFFF7
AREA_RXTOR	DOUBLE	-17	FFFFFFEF
ACTIV	DOUBLE	-25	FFFFFFE7
RE	DOUBLE	-33	FFFFFFDF
VEXT	DOUBLE	-41	FFFFFFD7
KM	DOUBLE	-49	FFFFFFCF
TOTL_VOLU	DOUBLE	-57	FFFFFFC7
DIFF	DOUBLE	-65	FFFFFFBF
AREA	DOUBLE	-73	FFFFFFB7
TORT	DOUBLE	-81	FFFFFFAF
VSP	DOUBLE	-89	FFFFFFA7
FARA	DOUBLE	-97	FFFFFF9F
DENS	DOUBLE	-105	FFFFFF97
ENZYME_IMM	DOUBLE	-113	FFFFFF8F
Z	DOUBLE	-121	FFFFFF87
CSOU	DOUBLE	-129	FFFFFF7F
ETA	DOUBLE	-137	FFFFFF77
TOTL_AREA	DOUBLE	-145	FFFFFF6F
THEILE	DOUBLE	-153	FFFFFF67
PSI	DOUBLE	-161	FFFFFF5F
VMI	DOUBLE	-169	FFFFFF57
BETA	DOUBLE	-177	FFFFFF4F
VFLU	DOUBLE	-185	FFFFFF47
CSIN	DOUBLE	-193	FFFFFF3F
PIE	DOUBLE	-201	FFFFFF37
DIFFE	DOUBLE	-209	FFFFFF2F
NUM_BEADS	DOUBLE	-217	FFFFFF27

User functions arguments and locals

Name	Type	Offset(R10)

FN.TANH	DOUBLE	119	00000077
X	DOUBLE	111	0000006F

Final dynamic offset	-217	FFFFFF27
Total variable space	344	00000158
Stack space in pages	1	00000001

PROGRAM SECTIONS

	Name	Bytes	Attributes
0	$PDATA	490	PIC CON REL LCL SHR NOEXE RD NOWRT LONG
1	$CODE	1456	PIC CON REL LCL SHR EXE RD NOWRT LONG

EXTERNAL REFERENCES

OTS$LINKAGE	BAS$LINKAGE	BAS$POWDD	BAS$INIT_R8
BAS$INIT_DEF_R8	BAS$END_R8	BAS$END_DEF_R8	BAS$INPUT
BAS$PRINT	BAS$IO_END	BAS$IN_D_R	BAS$OUT_D_V_S
BAS$OUT_D_V_B	BAS$OUT_T_DX_S	BAS$OUT_T_DX_B	BAS$OUT_T_DX_C
MTH$DEXP_R7			

LINE NUMBER CROSS-REFERENCE TABLE

Line	References
250	800.003
1000	800.003

SYMBOL CROSS-REFERENCE TABLE

Symbol	Class	References			
ACTIV		80.001	200.001		
AREA		41.001	77.001		
AREA_RXTOR		73.001	195.001		
BETA		440.001	460.001		
CINF		200.001	210.001	460.001	
CSBL		75.001	200.001		
CSIN		520.001	630.001		
CSOU		250.001	460.001	520.001	
DENS		30.001	420.001	430.001	
DIFF		10.001	47.001	430.001	440.001
DIFFE		47.001	610.001		
ENZYME_IMM		72.001	110.001		
ETA		620.001	622.001	640.001	
FARA		5.001	510.001		
FN.TANH	F	500.001#	501.002	501.003	620.001
KCAT		100.001	110.001		
KM		60.001	610.001	630.001	
LAMBDA		510.001	520.001		
MWENZ		90.001	100.001	110.001	
MWHEP		85.001	200.001		

NUM_BEADS		76.001	77.001	78.001			
PIE		1.001	41.001	42.001	73.001		
PSI		45.001	510.001				
R		4.001	510.001				
RADI		40.001	41.001	42.001	420.001	440.001	610.001
RE		420.001	440.001				
SC		430.001	440.001				
T		2.001	510.001				
THEILE		610.001	620.001				
TORT		46.001	47.001				
TOTL_AREA		77.001	465.001				
TOTL_VOLU		78.001	110.001	640.001			
VEX		465.001	470.001	800.001			
VEXT		460.001	465.001				
VFLU		70.001	190.001				
VINF		190.001	195.001	420.001			
VINT		640.001	650.001	800.001			
VISC		20.001	420.001	430.001			
VM		50.001	100.001	610.001	630.001		
VMI		110.001	120.001				
VOLB		42.001	76.001	78.001			
VOLUME_BED		71.001	76.001				
VSP		630.001	640.001				
X	L	500.001#	501.001	501.003			
Z		86.001	510.001				

Key for special characters above:

```
+-----------------------------------+
!                                   !
!   C = Constant                    !
!   E = External                    !
!   F = user defined Function       !
!   I = subscripted ( Indexed )     !
!   L = Local parameter to function !
!   M = Map or common name          !
!   S = Subprogram name             !
!   V = Virtual array               !
!                                   !
!   # = explicit definition         !
!                                   !
+-----------------------------------+
```

Compilation qualifiers in effect

```
/CHECK=(BOUNDS,OVERFLOW)
/DEBUG=(NOSYMBOLS,TRACEBACK)
/LONG  /DOUBLE  /SCALE=0  /LINES  /SETUP
/OBJECT  /LISTING  /NOMACHINE  /CROSS
```

APPENDIX D

Effects of pH

This section is taken from my 24 unit lab report submitted to the Department of Biology in April, 1981 for partial fulfillment of the degree of Bachelor of Science. This information is included here for the readers' convenience.

It was desired to find the pH optimum for the free enzyme. This experiment was performed in two parts: 1) finding at what pH the enzyme was most active, and 2) finding at what pH the enzyme was most stable. The pH for maximum stability would then be used for storage of the enzyme. The pH giving a maximum activity with a sufficient stability for the enzyme to perform its function could be used in the heparinase reactor.

Using NAM buffer, the effects of pH are more easily assayed since NAM has buffering capacity from pH 2.0 - 11.0 and gives a linear titration curve.[124] The combined optimum activity and stability were determined by following the action of heparinase on heparin at three time points at various pH's from 3 to 11. The reaction was assayed at the same pH which was being examined.

Procedure: Fifty microliters of HA purified enzyme were diluted into 800 ul of NAM buffer at the desired pH. Twenty-five mg/ml solution of heparin in NAM were also made at each pH. The pH was adjusted in each case with either 10 N NaOH or concentrated HCl. All of these tubes were kept at 30°C +/- 1°C.

At times of 0, 1, and 4 hours, 200 ul of enzyme solution was combined

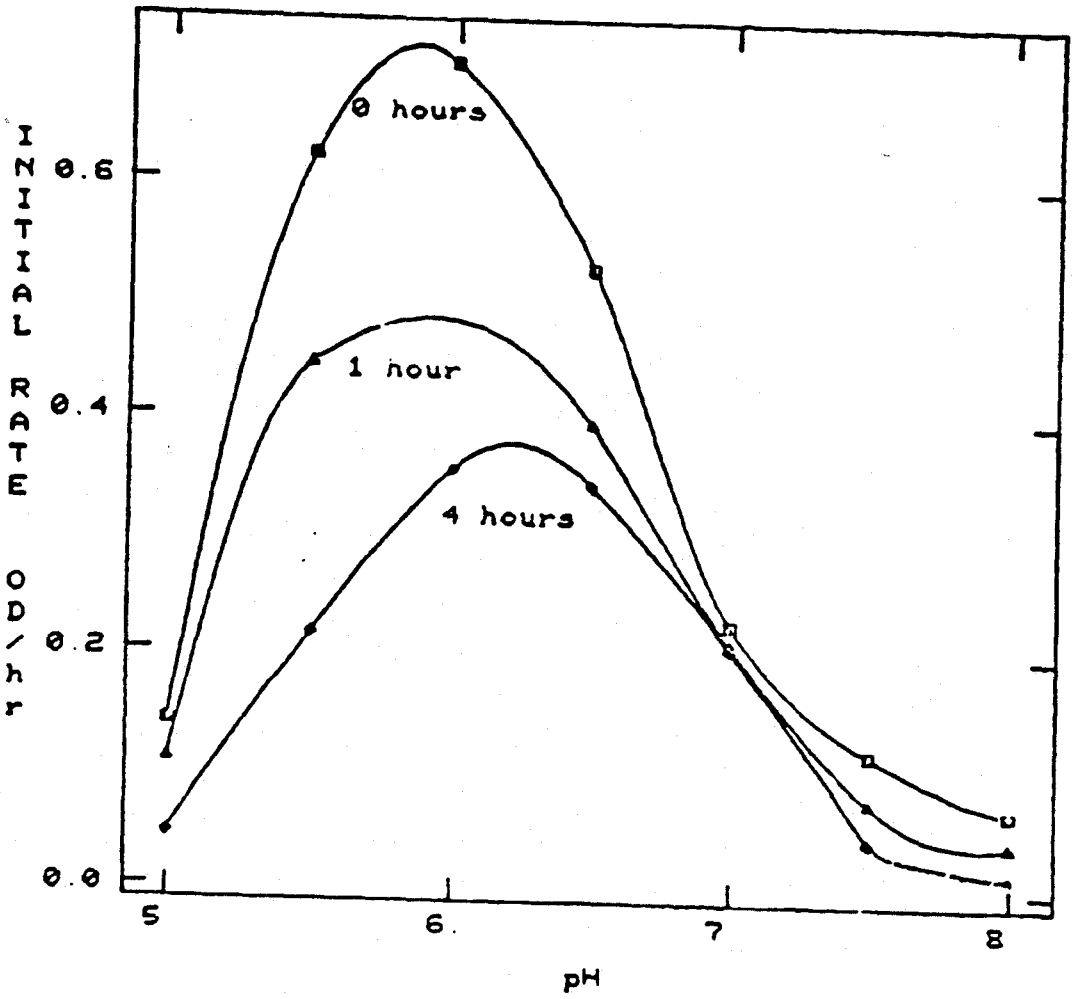
---

124.Linhardt, Robert J., unpublished results.

with 100 ul of heparin solution at the same pH and the reaction was assayed by UV 232nm.

Results and Discussion: the activity of heparinase at pH's from 5 to 8 is shown in Figure D-1. There is no activity shown by the enzyme beyond these limits. The maximum activity is a broad peak around pH 6. From the data taken over time at the same pH, the half-life denaturation of the enzyme is computed at each pH. Figure D-2 shows a maximum stability at pH 7. Flanagan has found that if the pH is brought back to 7.0 before assaying, the stability is very good toward high pH, and very poor at pH lower than 6.[125]

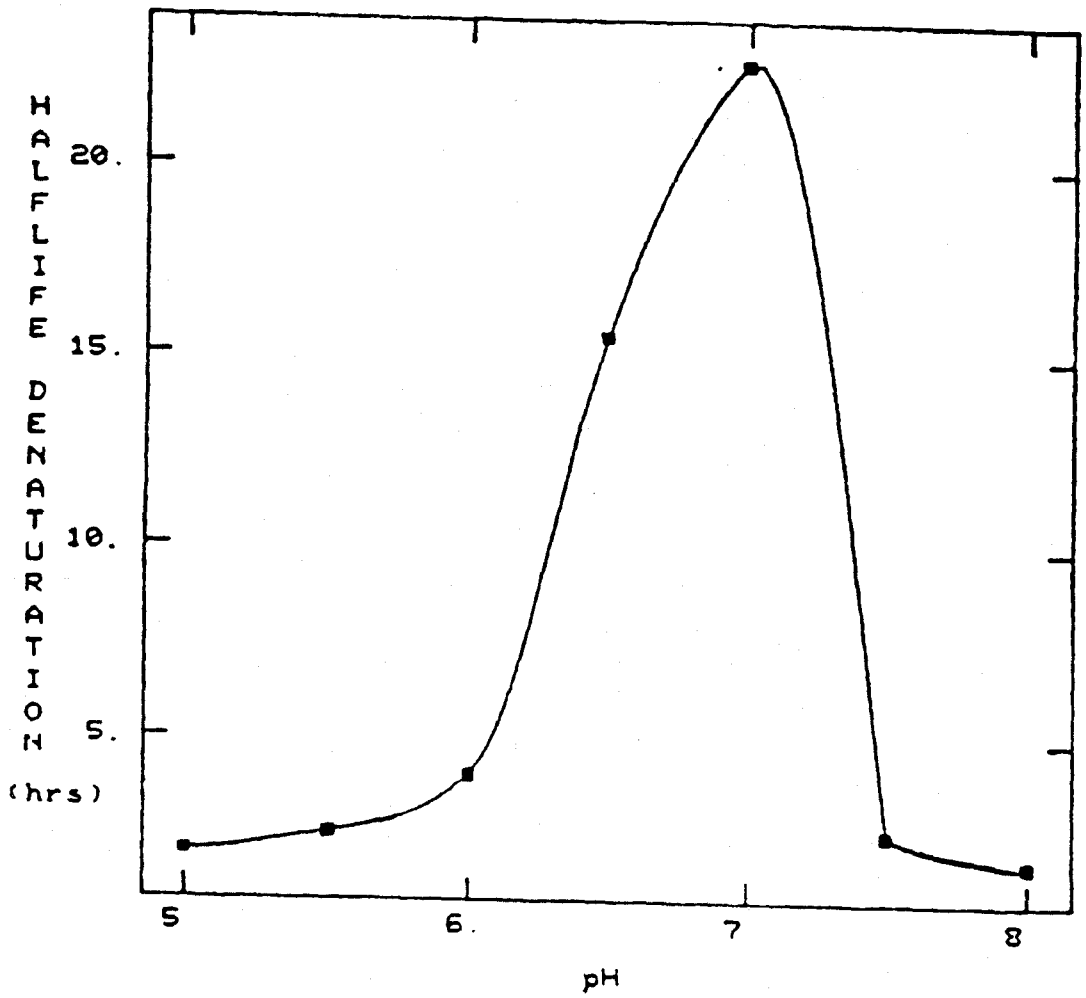
125.Flanagan, M.M., unpublished results.



Activity vs pH
Native Enzyme

FIGURE D-1

The activity of heparinase at pH's from 5 to 8 in NAM at 30°C is shown.



STABILITY vs. pH

Native Enzyme

FIGURE D-2

The stability of heparinase at pH's from 5 to 8 in NAM at 30°C is shown.

APPENDIX E

Cation Inhibition Screening

This section is taken from my 24 unit lab report submitted to the Department of Biology in April, 1981 in partial fulfillment of the requirements for the degree of Bachelor of Science. This information is included here for the readers' convenience.

A potential problem with an enzyme that is to be used within the body, especially in the blood, is the chance that it could be inactivated by trace amounts of metal cations. The cations were tested in NAM with the salt to be tested added at concentrations of 1×10^{-1}, 1×10^{-3}, and 1×10^{-5} M, wherever solubility of the cation permitted. The chloride salts of each cation were used except for zinc and lead, where the acetates were used, and molybdenum, where phosphomolybdic acid was used.

Procedure: Serial dilutions of the salts were made in both distilled water and NAM to get around solubility limitations in NAM. The salt solutions used are NAM plus salt at 2×10^{-1}, 2×10^{-3}, and 2×10^{-5} M. Reaction tubes are prepared with 200 ul of NAM with heparin at 25 mg/ml, 300 ul of NAM plus salt, 75 ul of NAM and 25 ul of HA purified enzyme. All tubes were pH balanced to pH 7.0. The temperature was controlled by a water bath at 30 °C +/- 1 °C. The assay method used was UV 232.

Results and Discussion: Table E-1 shows that at 1×10^{-5} M, almost all of the ions tested show little or no inhibitory effects on heparinase. The exceptions are Ca^{++}, and Hg^{++}, which show inhibition at both 1×10^{-5} and

1×10^{-3} M. Calcium is normally present in the blood at 5×10^{-3} M and will quite probably reduce the effectiveness of heparinase.

Table E-1. Cationic Inhibition Screening.

Concentration:	$1x10^{-1}$ M	$1x10^{-3}$ M	$1x10^{-5}$ M
Percent activity:			
Ca^{++}	i	50%	25%
Fe^{++}	i	i	100%
Fe^{+++}	i	i	100%
Zn^{++} (OAc)	i	i	100%
Cu^{++}	i	i	100%
Mo^{+6} (phosphomolybdic acid)	*	100%	---
Co^{++}	i	i	100%
Mn^{++}	i	i	100%
Sn^{++}	i	75%	100%
Cd^{++}	i	i	100%
Pb^{++} (OAc)	i	i	75%
Li^{+}	100%	100%	---
K^{+}	75-100%	---	---
Hg^{++}	---	0%	0%
Mg^{++}	100%	---	---
$NH4^{+}$	50%	100%	75%
Al^{+++}	i	i	100%
Ba^{++}	i	100%	75%

Key:

i - insoluble in NAM at this concentration

* - interferes with the UV 232nm assay at this concentration