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The relationship of carbohydrates and peptides in human fibrinogen and fibrin

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BOSTON UNIVERSITY

Dissertation

THE RELATIONSHIP OF CARBOHYDRATES AND PEPTIDES
IN HUMAN FIBRINOGEN AND FIBRIN

by

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PROBLEM

- a) To determine whether ester-like linkages are present in the primary, secondary, or tertiary structures of human fibrinogen and fibrin, and whether such linkages are related to the carbohydrate moieties of fibrinogen.
- b) To demonstrate and attempt to characterize the carbohydrate and peptide changes occurring during the conversion of human fibrinogen to fibrin by the action of human thrombin.
- c) To study some of the effects which ϵ -amino caproic acid, heparin, and other agents have on the conversion of human fibrinogen to fibrin. To evaluate whether the effects of heparin are related to the enzymatic activity of human thrombin or to the polymerization of fibrin.

BACKGROUND

I. HISTORICAL

a) Preparation of Fibrinogen

The coagulation of blood is one of the vital processes of life observed since biblical time. The entire process of transforming the free-flowing blood into a stable mechanical structure is initiated instantly and is generally regarded as completed within six to eight minutes. The major protein substance involved in blood coagulation was first isolated by Malpighi (1686) who described a white fibrous substance obtained from the red blood clot through washing with water. Malpighi also observed that neutral salts would retard or completely inhibit the coagulation of blood. Later, Nasse (1836) showed that fibrinogen could be salted out of plasma by means of sodium sulfate or saturated sodium chloride and upon dilution with water would be transformed into fibrin. Denis (1859) also suggested the existence of water-insoluble globulins (euglobulins) in plasma. Thirteen years (1872) later Alexander Schmidt formed the basis of our modern concept of blood coagulation and initiated the principle of precipitating fibrinogen and other globulins by dilution with slightly acidic water. In 1880 Hammarsten prepared horse fibrinogen by repeated precipitation with half-saturated sodium chloride, thereby establishing the first major method of plasma protein purification. He also reported that fibrinogen does not spontaneously coagulate but could be irreversibly heat denatured (+52 to + 55⁰C.) and that

fibrinogen and fibrin contain 16.66 and 16.91 per cent nitrogen, respectively. His nitrogen conversion factor (5.91) for fibrin is still acceptable. It would appear that some of Hammarsten's fibrinogen preparations were over 90 per cent pure. A series of modifications of Hammarsten's fibrinogen purification procedure were made by Rettger (1909), Howell (1910), Bardet (1920), Florkin (1930), Eagle (1935) and Chargaff and Bendick (1943). All of these modifications were directed at increasing the stability of the fibrinogen preparations, by the absorption of prothrombin on calcium triphosphate, calcium fluoride, barium phosphate or sulfate and magnesium hydroxide.

At the end of the 19th century a second major method of fibrinogen purification through the use of ammonium sulfate solutions was introduced and studied by Hofmeister (1899) and his many associates. McLean (1920), Smith et al (1934), Milstone (1941), Astrup and Darling (1942), Laki (1942), and Bagdy (1949) further studied the use of ammonium sulfate and were able to obtain a 75 to 80 per cent clottable fibrinogen. Laki and Bagdy reported the crystallization of swine fibrinogen; however, the clottability of Laki's preparation was less than 80 per cent, and Bagdy did not cite the clottability of his crystalline preparations. Laki continued to modify his original procedure, and in 1951 reported that by the proper control of pH, ionic strength and temperature, followed by two ammonium sulfate precipitations at 0.21 and 0.25 saturation, a bovine or human

fibrinogen preparation could be obtained which was 95 per cent clottable. The Laki purification procedure with ammonium sulfate yields a fairly stable produce (Ware and Lanchantin, 1954) which can be prepared free of plasmin (Katz et al, 1952). However Seegers (1955) claims that the Laki procedure yields a fibrinogen preparation which frequently contains blood coagulation components.

A third major method of fibrinogen purification was described by Ware, Guest and Seegers (1947) who obtained a 97 per cent clottable bovine fibrinogen. Their technique was based upon the fact that fibrinogen, unlike the other major plasma proteins, has a low solubility at low temperatures. By repeatedly freezing and thawing of plasma they were able to centrifuge out purified fibrinogen, and discard the other soluble plasma proteins.

The fourth approach at fibrinogen purification was introduced in the early 1940's by the Harvard group under the direction of E.J. Cohn. They were able to elaborate a now well known cold ethanol-water variable system for the fractionation of plasma proteins. The first fraction (I) of Cohn's method 6 (1946) contains about 80 per cent of the human plasma fibrinogen. The further purification of fraction I was described by Morrison, Edsall and Miller (1948) and their preparation contained between 93 and 98 per cent coagulable protein when treated with thrombin. However, upon ultra-centrifugation, even their purest preparations contained two sedimenting components ($S_{20} = 8.55$ and 15) and sometimes a more

rapidly sedimenting component. Blomback["] in a definitive thesis dissertation described the preparation of bovine and human fibrinogen from Cohn Fraction I. He and his wife (1956) utilized various molar concentrations of glycine to raise the dielectric constant of the medium and thereby increase the solubility and decrease interactions between most of the contaminating proteins in fraction I. Their preparations designated as I₄ were 98 to 100 per cent clottable. They were unable to detect prothrombin, thrombin, plasmin, anti-hemophilic globulin, cold insoluble globulins, or heparin cofactor in their preparations and their I₄ preparations were found to contain about 10 per cent of the plasminogen and pro-activator which was originally present in crude fraction I. Their preparations were electrophoretically homogeneous and had a sedimentation value of 6.50 S at pH 8.0, $f_{20} = 0.3$, and sedimented as a single peak throughout an entire one hour run. Mossessen (1962) utilized lysine and ϵ -aminocaproic acid to solubilize plasminogen and obtained a "plasminogen-free" fibrinogen using the Blomback["] I₄ fraction as the starting material. Since lysine and ϵ -aminocaproic acid are both potent inhibitors of plasmin, the preparation of Mossessen cannot be said to be entirely free of plasminogen. Many personal communications have confirmed the opinion of this investigator that the Blomback["] or Mossessen methods of preparing human fibrinogen represent the purest fibrinogen product which can be currently produced.

A fifth method for fibrinogen preparation was introduced in

England by Kekwick, et al (1946). They were able to obtain fibrinogen preparations which were at least 97 per cent clottable (Kekwick, et al, 1955). However, the yields are somewhat lower than the ethanol fractionation procedure, but the final product appears to be about as pure as that described by Morrison et al (1948).

b) Fibrinopeptides

The purest of fibrinogen when converted into fibrin by the action of thrombin was always found to leave some proteinaceous substances in the supernatant. Hammarsten (1880, 1896) and Schmidt (1892) referred to these protein substances as "fibrino-globulins". In 1908 Mellanby suggested that fibrinogen is composed of two associated proteins and that during coagulation the bond joining these groups is broken where upon a quantity of "fibrino-globulins" or serum globulins remain in solution. Waldschmidt-Leitz et al (1928) suggested that thrombin could be a "trypsin-like" enzyme since natural and synthetic trypsin substrates produced inhibition of the coagulation of whole blood. In 1937, Eagle showed that some snake venoms (*Bathrops jararaca*) and papain, which both contain proteolytic enzymes, could bring about the direct conversion of fibrinogen into fibrin. A year later Presnell demonstrated that the total nitrogen in fibrin formed by the action of thrombin was less than the corresponding amount of heat-coagulated fibrinogen. He also demonstrated by formal titration that the number of free amino groups was increased during the coagulation of

fibrinogen. Van Slyke (1941) was aware of the ninhydrin-reacting materials in picric acid filtrates of plasma and serum. Because of the variable results obtained with serum filtrates, the plasma was recommended for the Van Slyke amino acid analysis. In a simple, yet now classical experiment, Lorand (1951, 1952) demonstrated that during the fibrinogen-fibrin conversion by thrombin, there is a sharp rise in non-protein nitrogen which levels off at about 3 per cent of the total nitrogen present. From 1952 to 1958 a series of several articles appeared separately by Bailey, Bettelheim and Bailey (1952), Lorand and Middlebrook (1952, 1953), Bettelheim (1956), Blomback and Yamashina (1958) and Blomback and Vestermark (1958), which adequately described the amino acid composition, paper electrophoretic behavior, molecular weight, and resin column characteristics of bovine fibrinopeptides. During the fibrinogen-fibrin conversion two moles each of fibrinopeptides A and B per mole of fibrinogen were found. The amino acid sequence of these bovine fibrinopeptides suggested by the above mentioned workers have been confirmed by Blomback et al. (1959), Gladner et al. (1959) Folk and Gladner (1960) and Sjoquist (1960). The fibrinopeptides A (mol. wt. 1,890) was described as containing 19 amino acid residues. Two residues of serine and no tyrosine were found in the hydrolysates of fibrinopeptide A. The fibrinopeptide B (mol. wt. 2,460) was described as containing 21 amino acid residues. One tyrosine and no serine residues were found in the acid hydrolysates of this fibrinopeptide. The tyrosine is present in the fibrinopeptide B as tyrosine-O-sulfate ester (Bettelheim, 1954). This form of tyrosine ester

is unique in that it has not been demonstrated in any other mammalian protein. The proposed structures of the bovine fibrinopeptide are given below:

PEPTIDE A.

Glu (Asp₃-Ser₂-Pro₂-Gly₂)-Ala-Leu-Thr-(Glu-Gly₃)Val-Arg

M.W. = 1,890

PEPTIDE B.

N-acetyl-Thr-Glu-Ala-Pro-Asp-Tyr^{SO₄}-Asp-Glu-Gly-Glu-Asp₂-Arg-
Pro-Lys-Val-Gly-Leu-Gly-Ala-Arg

M.W. = 2,460

All of the information thus far presented has applied to the bovine fibrinogen-fibrin conversion system. The human system has thus far not been thoroughly investigated with regard to the nature of the fibrinopeptides. This dissertation represents a modest beginning directed at some of the marked differences in the human and bovine systems. These differences were so great that repeated experiments were necessary to confirm them, and at times modifications of present procedures had to be used which lengthened an otherwise simple analytical approach.

II. Evidence of Primary Carbohydrate-Protein Structures with Ester-Linkages.

The proteins used in the experiments of this dissertation have not been previously studied for ester-like linkages. However, the following proteins have been studied and a brief summary of the

results is presented here. These studies have contributed some of the methodology used in this dissertation. Furthermore some analogy between diverse proteins might be anticipated in regards to the protein-carbohydrate linkages.

a) Collagen

Crystallized connective tissue proteins (procollagen and collagen) contain hexoses (0.42 to 2.71%) and three types of coordinate bond linkages between carbohydrate and protein have been suggested by Grassmann et al (1957). (1) N-glycosidic linkages between sugar and amino groups of the protein. (2) O-glycosidic linkages to the hydroxy groups of hydroxyamino acids. (3) Ester-linkages between the alcoholic hydroxy groups of sugars and the carboxyl groups of the protein. Grassmann, Endres, and Steber (1954) demonstrated early evidence of such ester-linkages in procollagen by the formation of alcohols following treatment with lithium borohydride. The lithium borohydride reaction with collagen led Grassmann (1955) to the isolation of a gluco-peptide, glucosaminyl-leucyl-diglycyl-alaninol. A review by Fraenkel-Conrat (1956) discusses the point as to whether the C-terminal amino-acids of collagen are esterified or free.

A series of papers by Gallop, Seifter, Franzblau et al (1959, 1960, 1962) has presented more evidence that glucose is the alcohol donor of the ester bonds in ichthyocol (collagen derived from fish swim bladder).

Their group employed the hydrazine and hydroxamate reactions for the determination of esters and concluded that two or three ester bonds are linked through the alpha-carboxyl group of aspartic

acid and that the beta-carboxyl groups of asparatic acid participate in the other three ester bonds. They suggest eight ester bonds per molecule of ichthyocol (or, 6 per 1,000 amino acid residues).

After reviewing the series of papers presented by the Gallop group, the writer was impressed by the lack of information regarding the nature of the carbohydrates and the amounts which were present in the commercial gelatin preparations which they also studied. One can only surmise that at least glucose and galactose in the ratio of 1:1 are present in ichthyocol. (Loeven, 1955; Schneider, 1949). The earlier reports from the Gallop group presented evidence of the "ester-like" or perhaps imide linkages between units of amino acid chains of about 20,000 molecular weight. In view of their latest conclusions regarding the glucosidic linkage, one may postulate that the molecule of an alpha-unit of procollagen consists of three low molecular weight "protein backbone" units held together or interspaced with ester-linkages to the disaccharide lactose. Bello (1960) and Hormann (1960) suggested that "ester-like" bonds are involved in intermolecular and intramolecular cross-linkages. de la Burde, Peckham, Vies (1963) indicated that the hydrazine reaction employed by Gallop et al may be the result of an early reaction due to deamidation and later to deguanidination.

b) Ovine Submaxillary Mucoprotein (OSM)

A classical series of reports on ovine, submaxillary mucoprotein (OSM) by Gottschalk, et al (1959, 1960, 1960_a, 1961) indicated a convincing demonstration of ester-linkages. The mucoprotein OSM contains 42%

hexoses, consisting of equimolecular amounts of N-acetyl neuraminic acid and N-acetyl galactosamine, a high percentage of amino dicarboxylic acids (10.7) and only 0.33% total amide nitrogen. One's attention is drawn to the numerous and elegant approaches which these investigators used to demonstrate ester bonds in OSM. First, they used crystalline enzymes as analytical reagents for the partial hydrolysis of proteins or polypeptides and disaccharides. Secondly, they used the most appropriate conditions under which lithium borohydride will reduce the ester groups to their corresponding alcohols, (but will not react with the acid amide groups and with less than 2% of the total peptide bonds of proteins). The conclusions of the Gottschalk group concerning the mucoprotein fragment from OSM indicate that about 80% of the prosthetic groups are involved in a glycosidic-ester linkage to the free carboxyl groups of aspartyl and glutamyl residues, and that about 18% of the total N-acetyl neuraminic acid in OSM is bound by an O-glycosidic linkage to the hydroxyl groups of serine and/or threonine residues. Unlike the carbohydrates involved in collagen, those found in the prosthetic group of OSM are disaccharide units of α -D-N-acetylneuraminyl (2 \rightarrow 6) N-acetylgalactosamine. In other words, the protein core of OSM has about 80% of the carboxyl groups of aspartic and/or glutamic acids involved as ester linkages with the hydroxyl groups of the disaccharide units (probably through the N-acetylgalactosamine moieties, since the N-acetyl neuraminic acids are the terminal units of OSM). The remaining 18%

of the disaccharide units are covalently linked to the OSM protein core by stable O-glycosidic linkages with serine and/or threonine. Furthermore, the OSM protein core probably consists of α -helix segments "loosely-held" by proline residues, and the disaccharide units forming a secondary incomplete helix around the protein core.

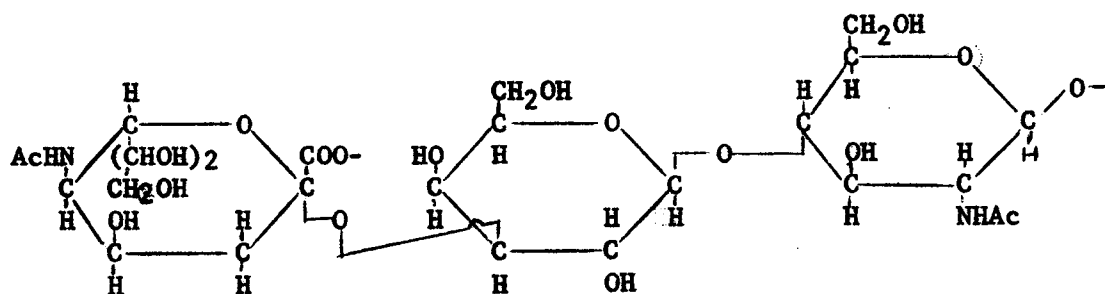
c) Ovalbumin

There have been to date three glyco-peptides isolated from tryptic digests of crystalline ovalbumin (Cunningham, 1957; Jevons, 1958; and Johansen, 1958). The ovalbumin used in the latter two of these experiments was that prepared by Neuberger, (1958) which contained 1.8% mannose and 0.9% glucosamine. The carbohydrate-amino acid structures of these three glyco-peptides are remarkably similar in that each contain mannose:glucosamine (N-acetyl glucosamine):aspartic acid:leucine in proportions approximately of 4:2:1:1. The other amino acids recovered were serine and threonine. Upon further digestion of the glyco-peptide with carboxypeptidase, Johansen obtained a carbohydrate-containing fragment with only one mole of aspartic acid and trace amounts of threonine and serine, and suggested that one carboxyl of aspartic acid was linked with carbohydrate and the other carboxyl of this amino acid was linked to leucine. He found no experimental evidence to support the existence of O-glycosidic linkages involving the hydroxyl groups of threonine or serine as originally suggested by Werner (1953). A most recent investigation by Lee and Montgomery (1962) indicates that ovalbumin contains a single unit of oligosaccharide with the end amino acid-carbohydrate sequence being glu-lys-tyr-asp-carbohydrate.

d) Fetuin

R.G. Spiro (1960, 1962) isolated a glycoprotein of fetal calf serum (fetuin) and subjected it to digestion with various proteolytic enzymes (pepsin, chymotrypsin, trypsin, nagarse, and papain). As a result of DEAE-cellulose chromatography of the pepsin and nagarse digest of fetuin, he was able to obtain seven distinct glycopeptide residues which contained all of the sugar in the same molar ratio present in the original fetuin. He stated that the most likely composition of the polysaccharide units of the glycopeptides would be 4 residues of sialic acid, 4 residues of galactose, 6 residues of hexosamines, and 3 residues of mannose. The amino acids most frequently occurring in the vicinity of the three carbohydrate-peptide linkages were aspartic acid, alanine, serine and proline. Eylar (1962) suggested that the linkages between amino acids and carbohydrates were through the reducing groups of N-acetylglucosamine to the beta-carboxyl group of aspartic acid. The diagrammatic representation of the carbohydrates and their linkages is presented here with permission of Dr. Spiro (1962).

Basic Oligosaccharide chain of fetuin.



N-acetyl neuraminic acid - galactose - N-acetyl glucosamine

albumin, a crystalline product was obtained by Kendall (1941) and Cohn et al (1947) which was reported as carbohydrate-free (as tested by the orcinol reaction). According to Tristram (1953) the amino acid residue analysis of crystalline albumin has been said to account for 92.97% of its nitrogen. The treatment of crystalline human plasma albumin with mercuric chloride and resin fractionation has led to the preparation of an "albumin dimer" and mercaptalbumin (Allerton, 1961).

The solubility of human crystalline albumin appears to be influenced by the variable amounts of lipid (2.9 to 0.4%) which it contains. Crystalline mercaptalbumin preparations (Purdy, 1962) were found to contain the least amount of fatty acids (i.e. between 0.5 and 1.0 mole of fatty acid per mole of albumin).

b) Fibrinogen and Fibrin

Part of the studies reported below is devoted to the possible existence of ester-type linkages in fibrinogen and fibrin. There are several excellent reviews concerning the purification, physical chemical properties, and amino acid composition of fibrinogen and fibrin (Blomback, 1958; Scheraga and Laskowski, 1957; and Scheraga, 1961). Some of those characteristics of fibrinogen and fibrin which are pertinent to this study are presented here.

The carbohydrate content of the previously mentioned proteins (albumin, ovalbumin, fetuin, and OSM) is documented by many investigators. The nature of the di- and poly-saccharides and their linkages with the protein moiety is currently being investigated.

There are on the other hand only two reports concerning the total carbohydrate content of human fibrinogen and fibrin. According to Consden and Stanier (1952, 1953), human fibrinogen contains 4.6% reducing sugars and 1.1% hexosamine, while fibrin contains 2.7% reducing sugars and 0.6% hexosamine. Their fibrin was obtained directly from human plasma, and the purity of the fibrinogen used in their analysis was not indicated. The only other report, that of St. Szára and Bagdy (1953), indicated that there was $1.73 \pm 0.01\%$ hexoses. The fibrinogen used in these analyses was reported as 95-98% clottable, and was crystallized according to Bagdy's technique, although they give insufficient details of the crystallization procedure. They were unable to detect the presence of "sialic acids", yet Böhm and Baumeister (1955) were able to isolate 0.64 to 0.89% "neuraminic acids" from the fibrin of normal human plasma. The carbohydrates obtained by prolonged acid hydrolysis (110°C) were identified as galactose, mannose, and glucosamine. The uronic acids were reported as not present (Consden and Stanier, 1953). More details of the analyses of bovine fibrinogen and fibrin, taken from the papers of Bagdy and Blomback, are illustrated in the following table:

	hexoses (% dry wt.)	glucosamine (% dry wt.)	sialic acid (% dry wt.)
Bovine fibrinogen	1.64 ± 0.02	0.56 ± 0.02	none
Fibrin	1.33 ± 0.04	0.54 ± 0.01	none
Bovine Fibrinogen	3.2 ± 0.3	1.0	0.8
Fibrin	2.8 ± 0.2	1.0	0.8

Blomback and Blomback (1957) have indicated that the sialic acid in fibrinogen is of the ovine type. All of the above analyses indicated a 10-20% difference between fibrinogen and fibrin which suggests that carbohydrates release occurs during clotting. As indicated by Scheraga (1957), "thus far, this problem of the apparent liberation of carbohydrate has not been resolved".

IV. Discussion of Problem

Because human albumin lacks carbohydrates in its structure, it was chosen as the reference protein for this investigation of the carbohydrates and ester linkages of fibrinogen. If "ester-like" bonds were found in albumin they would be typical of intramolecular ester cross-linkages due to secondary or tertiary structure, rather than carbohydrate esters. Human α_1 -glycoprotein was chosen as the second reference protein because "ester-like" bonds found in this protein would suggest carbohydrate esters. There were no reports concerning the existence of "ester-like" bonds in either human albumin, α_1 -glycoprotein, or fibrinogen.

The second portion of the problem concerning the release of fibrinopeptides during the human fibrinogen-fibrin conversion has not been fully studied. However, the amounts of peptides and carbohydrates released during the bovine fibrinogen-fibrin conversion were clear from the following calculations: When 330 mgm. of fibrinogen are converted into fibrin, there are released about 9.9 mgm of fibrinopeptide nitrogen, and between 1.1 and 6.3 mgm. of

carbohydrates. Normal human plasma contains about 330 mgm. of fibrinogen per 100 ml.

A third problem, namely the unusual effects of heparin on the relatively purified coagulation system of thrombin and fibrinogen was made evident when it was observed that the effects of heparin were on the polymerization of fibrin. The problem was further complicated when it was observed that certain thrombin preparations when treated with small amounts of heparin would result in the increase of enzymatic activity of some thrombin preparations but not others.

EXPERIMENTAL

I. Alkaline-hydrazine Treatment of Various Plasma Proteins

a) Human Plasma Albumin

Three human plasma albumin (Fraction V) preparations, two crystalline plasma albumin preparations and a crystalline mercaptalbumin preparation were obtained through Dr. Pennell of the Protein Foundation or Dr. Oncley of Harvard University. All of these preparations were analysed to select the most suitable or purest preparation for total hexoses (orcinol method of Winzler, 1955). Protein nitrogen analyses were made by a Kjeldahl method on an air dry weight basis (Van Slyke and Hiller, 1948). The protein content was checked in two cases by U.V. $^{280 \text{ m}\mu}$ absorption in a Beckman D.U. using an extinction coefficient of 5.30 for a 1% solution of human albumin and in subsequent calculations the molecular weight was taken to be 69,000 (Cohn et al. 1947).

Results:

Preparation	% Hexoses*
Fraction V, Lot # HP-2	0.5
Fraction V, Lot # 1041	1.2
Fraction V, Lot # 1077	0.4
Crystalline albumin Lot # 1018	Less than 0.1
Crystalline albumin Lot # HP-2R	Less than 0.1
Mercaptalbumin	Less than 0.1

*Based on mgm. hexoses (mannose/galactose, 1:1) per 100 mgm. protein (Kjeldahl nitrogen)

Lot # 1041 was subjected to electrophoresis on cellulose acetate strips using barbital buffer, pH 8.6, /2 0.05. The strips were stained with light green S.F. dye in 1% acetic acid. The excess stain was eluted with 0.5% acetic acid; the fractions were

cut from the cellulose strip, and the protein dye dissolved in 0.5N NaOH. The eluted protein dye color was redeveloped with concentrated acetic acid and read at 625 m μ in a Coleman Jr., Model 6A. Lot # 1041 was found to contain 4.05% α_1 -glycoprotein. Similar findings for crude Fraction V were previously reported by Cohn E.J., et al (1947).

Lot # 1018 was found to be 76.74% protein by triplicate Kjeldahl and U.V.^{280 m μ} analyses, contained no detectable hexoses, and was electrophoretically homogeneous. 150 mgm. of this human albumin preparation was dissolved in 10 ml. of 1 Molar hydrazine at pH 9.0 and incubated in a 37°C water bath for 90 minutes. The unreacted hydrazine was separated and removed from the albumin by passing the mixture through a 55 x 220 mm. column (Scientific Glass # J-1661) of Sephadex G-50 (Lot #To 9860M) in the manner described by Porath and Flodin (1959). The column was eluted at room temperature (ca. 24°C.) with 1%(w/v) monochloroacetic acid (MCA) solution, pH 2.0, at the rate of 2.5 ml. per minute. The effective separation of the free hydrazine and albumin on this column shown in Figure I.

The samples containing the most protein (as determined by Kjeldahl nitrogen analysis) were examined for free and heat-labile (80°C.) hydrazine in the manner described by Gallop, et al (1960). An equal concentration (3.84 mgm./ml.) of untreated crystalline albumin dissolved in 1% MCA was used as the appropriate blank solution. The results of two separate experiments (Lot # 1018 & HP-2R) showed

CHROMATOGRAPHIC SEPARATION OF
ALBUMIN OR FIBRINOGEN AND HYDRAZINE
ON A SEPHADEX G-50 COLUMN

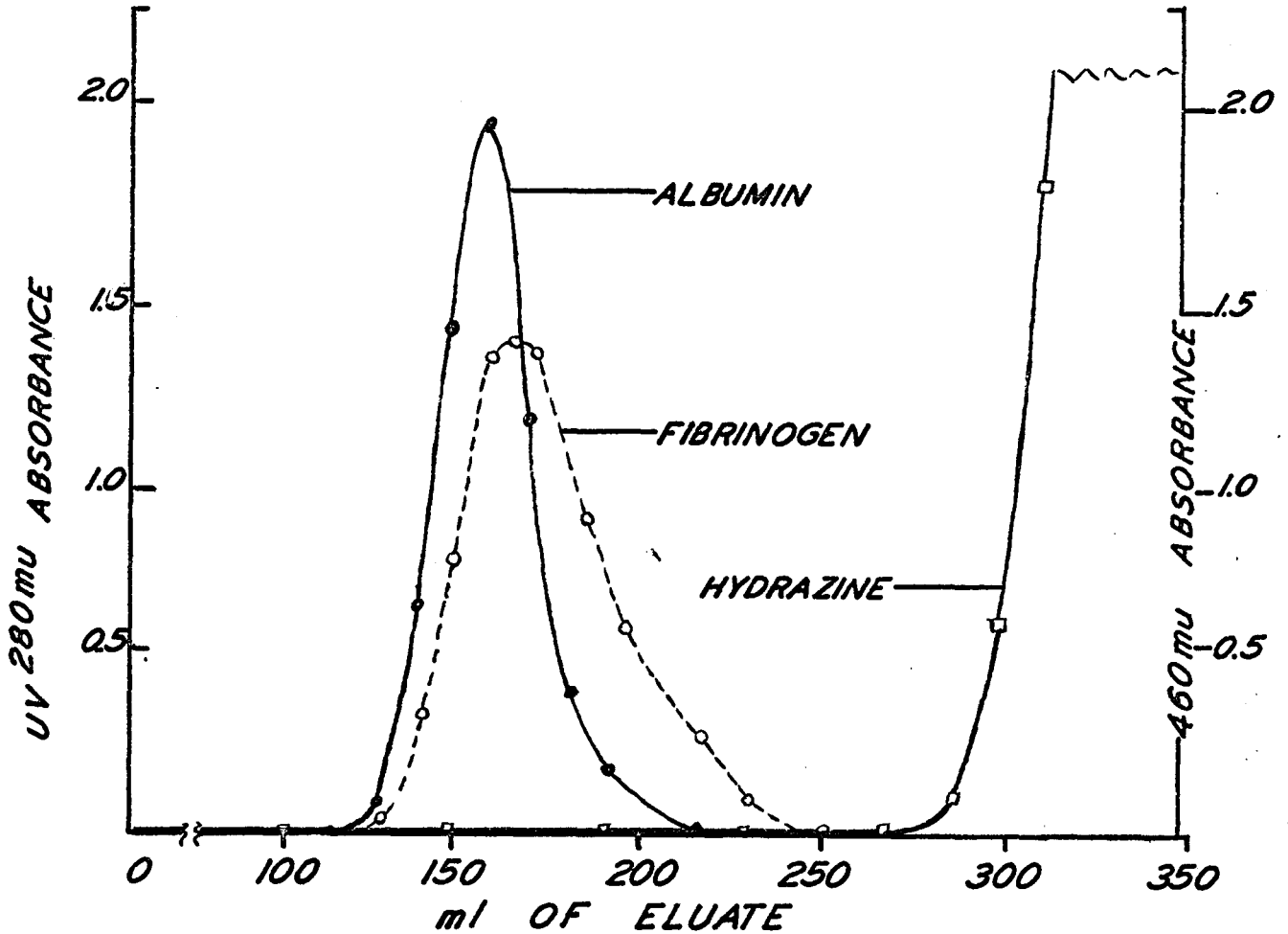


Figure 1. - The separation of human albumin or fibrinogen from unreacted hydrazine on a Sephadex G-50 column (55 x 200 mm.) at 24°C. with 1% (w/v) monochloroacetic acid at the elution rate of 2.5 ml./min. The protein UV^{280mu} absorbances were made with a Beckman DB. The 460 mu absorbances of the Ehrlich's reaction with hydrazine were made with a Coleman 6A.

identical column separation, and the mole ratio of the heat-labile hydrazine to albumin was found to be 0.20 and 0.28, respectively.

b) Crystalline Bovine Albumin

Two preparations of crystalline bovine albumin (Armour's Lots #V 68802 and W 69312) and one bovine mercaptalbumin preparation were used in these experiments. The U.V. extinction coefficient ($E_{1\text{cm}}^{1\%}$) at 280 mu for bovine albumin was taken as 6.60 and the nitrogen conversion factor of 6.23 was used (Brand, 1946). The molecular weight used was taken as 65,000 (Creeth, 1952).

Samples of 150 mgm. each of these protein preparations were treated with buffered hydrazine in the manner previously described for human plasma albumin. The Sephadex G-50 elution characteristics were similar to those for the human plasma albumin preparation, and the mole ratio of heat-labile hydrazine to albumin was found to be 0.68 (Lot #V 68802) 0.65 (Lot # 69312) and 0.50 (mercaptalbumin).

c) Human α_1 -glycoprotein

This human plasma glycoprotein was chosen for its purity and the 17.2% carbohydrate content. The starting material Cohn fraction VI was further purified in the manner described by Schmid (1953). The analysis table given by Schmid was used in the calculations, i.e. 10.7% nitrogen and $E_{1\text{cm}}^{1\%}$ at 278 mu equal 8.93. The molecular weight was taken as equal to 44,100 (Weimer, et al, 1950)

When 137.25 mgm. of this protein was examined for heat-

labile hydrazine as previously described; a mole ration of heat-labile hydrazine to α_1 -glycoprotein was found to be 0.32.

d) Bovine Fibrinogen and Fibrin

The nitrogen conversion factors of 6.02 and 5.91 for fibrinogen and fibrin, respectively were used (Blomback, 1957). The extinction coefficient ($E_{1\text{cm}}^{1\%}$) at 280 mu for bovine fibrin and fibrinogen were taken as 15.91 (Ferry and Morrison, 1947). This value was found to be correct for both fibrin and fibrinogen in 1% MCA solutions. However, the extinction coefficients ($E_{1\text{cm}}^{1\%}$) at 280 mu for bovine fibrin and fibrinogen in alkaline urea were taken as 16.84 and 16.51, respectively (Blomback and Blomback, 1956). The discrepancy of the extinction values is due to the variance of tyrosine absorption in the acid and alkaline pH ranges, which have been demonstrated and studied by both Tanford et al (1955) and Wetlaufer (1962). Fauth and Ward (1962) in a preliminary report stated that the $E_{1\text{cm}}^{1\%}$ value of 282 mu for fibrinogen in 1N NaOH was 17.3. The molecular weight was taken as 340,000 for fibrinogen (Fritzgerald, 1957). The total hexoses were determined by the orcinol method, as previously cited. The coagulation of fibrinogen and the syneresis of the fibrin clot was conducted according to the method of Morrison (1947); although a higher concentration of bovine thrombin (20 NIH units/ml.) was used (Saifer and Newhouse, 1954). All stock bovine thrombin preparations were from Upjohn Company and stored at -20°C. in the dry powder form, being dissolved in distilled water just prior to their use.

The clottability of two preparations of bovine fibrinogen (Armour's Lots #W5880 and W6321) were 42.0 and 45.1% respectively. The average per cent total hexoses (3 analyses) in this fibrinogen and fibrin was 3.0 and 2.6, respectively.

When 100 mgm. of bovine fibrinogen (Lot #W6321) was treated for 30 minutes in 1M hydrazine at pH 7.8 and analyzed after 72-hour continuous dialysis, the ratio of heat-labile hydrazine to protein was found to be 1.72.

A pilot preparation of 5 grams of Armour's bovine fibrinogen was subjected to purification according to the method of Blomback (1958). A 35-40% yield of 95.5% clottable bovine fibrinogen was obtained. The expense of the starting material, the low yields, and the gross impurities of the bovine thrombin, warranted the further studies to be conducted on human fibrinogen.

II. Human Fibrinogen and Fibrin

The nitrogen conversion factors, U.V. extinction coefficients, clottability and molecular weight used were the same as those cited for bovine fibrinogen and fibrin. The purified human thrombin preparation (270 NIH units/ml.) was obtained by a Cellex-P column method and was kindly prepared and supplied by Drs. Fenickel and Inman of the Ortho Research Foundation. The human thrombin solution was said to be free of plasminogen and plasmin. This preparation of thrombin was examined for tyrosine content by the Lowry, et al (1951) phenol method, and was found to contain 4,320 NIH

units/mgm. tyrosine which agreed with the value of 4,100 NIH units/mgm. tyrosine found by Dr. Inman's (1962) laboratory. Seegers, et al (1956) has suggested that pure bovine thrombin may have an activity of at least 1,400 units/mgm. dry protein and has obtained preparations of 30,000 to 47,000 units/mgm. tyrosine (bovine thrombin contains 3.2% tyrosine). Human thrombin has not been consistently obtained with the activities given for the bovine preparations. During the course of this study two preparations were received in a frozen state and were then thawed, diluted with phosphate buffer, pH 6.4 $\Gamma/2 = 0.1$. One milliliter aliquots of these diluted samples containing from 0.27 to 54.0 NIH units/ml. were kept at -20°C . until they were used.

a) Preparation of Purified Human Fibrinogen and Total Carbohydrate Contents of Fibrinogen and Fibrin.

The human fibrinogen used in these experiments was prepared from crude Cohn fraction I; method 6. The fraction I lots # 507,508, and 509 were obtained through the courtesy of the Red Cross Fractionation Laboratory. The out-dated whole ACD collected blood used to prepare the crude fraction I was said to be processed between 30-60 days after the bleeding date. The average day age of the citrated whole blood was 45 days. One Lot # 508 was especially selected and prepared from 30-day old ACD blood. It was observed that the harvested crude fraction I contained variable amounts of hemoglobin. The hemoglobin appeared as variegated and striated

bands in the upper third of the centrifuged precipitate of fraction I. The lower portion appeared essentially free of hemoglobin and lots # 508 and 509 were prepared from the lower portions. The hemoglobin concentrations were not done, but a personal experience with solutions of oxyhemoglobin at various concentrations enables one to visually estimate the contamination to be about 500 mgm%.

The purified human fibrinogen (Fraction I₄) was prepared from the crude Cohn fraction I according to the method of Blomback and Blomback (1956). No attempts were made to obtain lyophilized preparations because previous reports indicated a loss of 1 to 5% clottability occurred during the freeze-drying procedure (Morrison et al, 1948). The stock fibrinogen fraction I₄ remained 97-100% clottable after two months storage at -20°C. The fibrinogen preparations were removed from the deep freeze for brief periods necessary for sample weighing. It was noted that clear ice-like crystals could be separated from the frozen fibrinogen fraction I₄ during each weighing period. The original fraction I₄ was found to contain 25% protein. By the removal or avoidance of the ice-like crystals, the fraction I₄ could be made to contain 40% protein. The reader is referred to page three of this dissertation describing the freeze-thawing method of fibrinogen purification.

The clottability and total hexoses were determined at the various precipitating stages during the fibrinogen purification and are presented in Table I. At Step I₂ where the lowest yield is

TABLE I

The total hexoses and clottability of various fibrinogen fractions in per cent of total protein.*

	<u>% Hexoses</u>	<u>% Clottability</u>
Cohn Fraction I (Fibrinogen)	2.7 - 2.0	41 - 53
Fibrin	2.0 - 1.7	
"		
Blomback Fraction E ₂ (Fibrinogen)	1.9	83 - 86
Fibrin	1.5	
"		
Blomback Fraction I ₂ (Fibrinogen)	1.6	94 - 97
Fibrin	1.3	
"		
Blomback Fraction I ₄ (Fibrinogen)	1.6	97 - 100
Fibrin	1.3	

* The carbohydrate components of fibrinogen and fibrin

	<u>% Hexoses**</u>	<u>% Hexosamines</u>	<u>% Sialic Acids**</u>
"			
Blomback Fraction I ₄ (Fibrinogen)	1.6 ± 0.1	0.6	1.0 ± 0.1
Fibrin	1.3 ± 0.1	0.6	0.9 ± 0.1

* The number of analyses, methods, and reference standards are given in the text.

** The ± indicates the range in % hexoses and sialic acids of all samples analyzed.

obtained (ca. 65%): the cold and insoluble globulin complexes were separated from the fibrinogen. These cold-insoluble globulins were found to contain 1.6% total hexoses, suggesting that they are probably denatured fibrinogen.

The average total hexoses (34 analyses), hexosamines (5 analyses), and sialic acids (27 analyses) values are also given for fraction I₄. The reference standards were galactose (glucose-free):mannose (1:1) for total hexoses by a modification of the orcinol-H₂SO₄ method described by Winzler (1955) and Vasseur (1948), and glucosamine hydrochloride by a modification of the p-dimethylaminobenzaldehyde method of Elson and Morgan (1933) as described by the Retina Foundation (1959), and N-acetyl neuraminic acid by the sialic acid method of Werner and Odin (1952). The N-acetyl neuraminic acid was kindly supplied by Dr. Fable Hansen (Retina Foundation), and was said to have an extinction value of 57,000 when tested by the thiobarbituric acid method of Warren (1959). All other reference standards were found to be chromatographically pure when examined by the method of Dimler, et al (1952).

b) Chromatographic Identification of the Sugars Present in Fibrinogen

For the detection of sugars other than sialic acids, 100-150 mgm. of either fibrinogen or fibrin were hydrolyzed in 5 ml. of 1.00 N H₂SO₄ for 18 hours at 105°C. in nitrogen flushed, evacuated, then sealed vials. The hydrolysates were titrated to

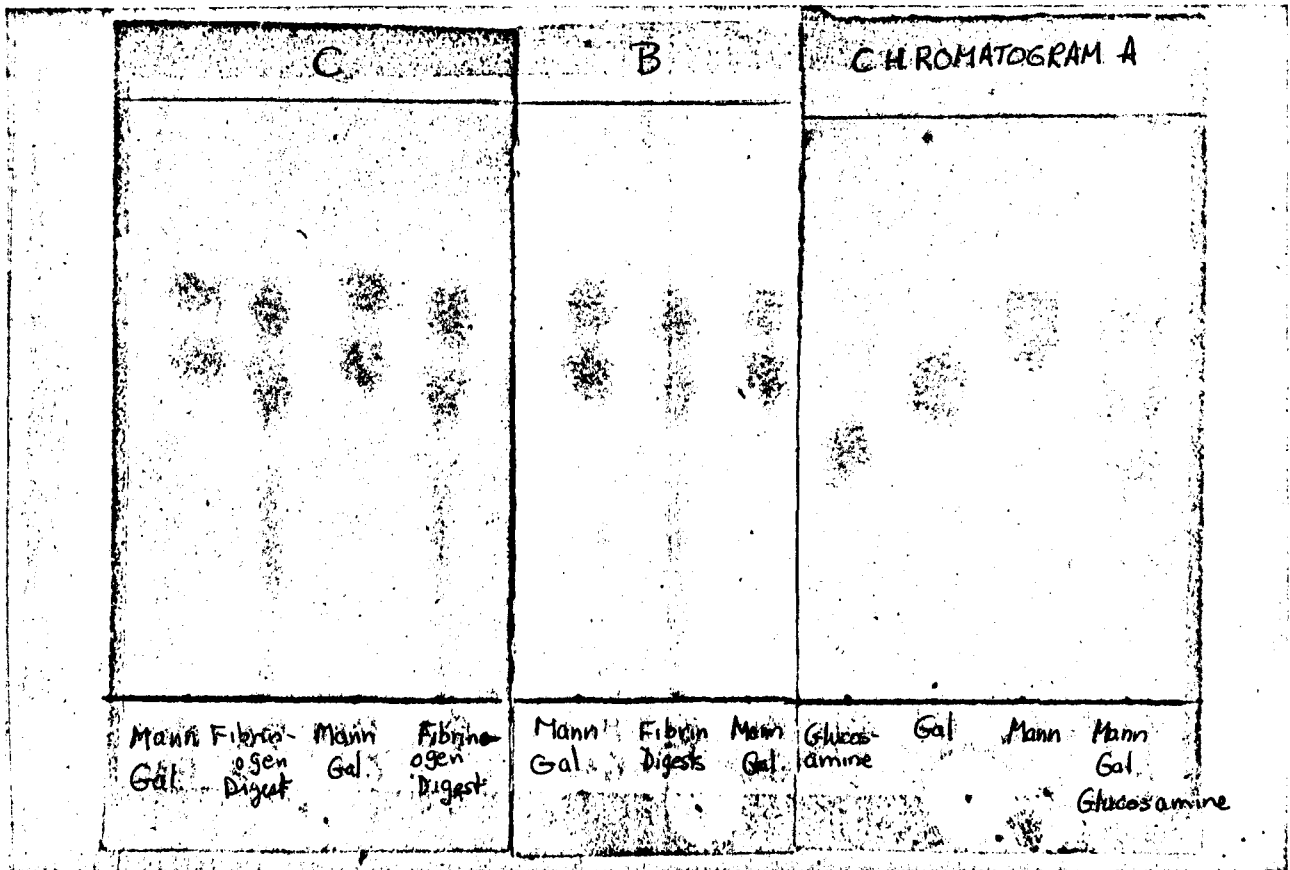
the pH 7.4 ± 0.1 with a saturated solution of barium hydroxide, and the excess barium precipitated by bubbling CO_2 into the suspension until a pH of about 5 was reached. The precipitate and most of the brown color were separated by dialysis against distilled water (4 volumes x 3) at 5°C . in Visking 23/32 dialysis tubing. A sealed, B-B shot-weighted glass tube was inserted into the dialysis tubing and used to increase the dialysis surface. A magnetic bar was previously placed in the bottom of the dialysis bag, and a continuous rotary mixing of the dialysate was achieved. The first dialysis was over-night, and each subsequent dialysis was for 3 to 4 hour periods. The combined dialysates, which were clear and nearly colorless were concentrated to dryness by freeze drying. The residue was dissolved in 0.5 ml. of distilled water and the flask twice washed with 0.5 ml. of distilled water aliquots. The dissolved material, and washings were applied to a column (0.75 x 20 cm.) of Dowex 50-X2, 200-400 mesh (hydrogen form). The sugars except hexosamine were eluted from the column with four column volumes of distilled water. The eluates were concentrated to a dry residue as above and dissolved in 100 lambda of distilled water. Aliquots of 1 to 5 lambda were used for unidimensional multiple paper chromatography according to a modification of the method of Dimler, et al (1952). Thin layer chromatography (350 u thickness) was equally effective with the following mediums: cellulose phosphate, carboxymethyl cellulose, diethylaminoethyl cellulose, Kieselguhr G, and Silica Gel. All of

these were used 10% CaSO₄ as a binder, except in the case of carboxymethyl cellulose where a 15% CaSO₄ binder was found to give the most uniform plates. The chromatographic solvent developer found most useful in both chromatographic methods was n-butanol: pyridine:water (6:4:3 by volume). One redevelopment with the same solvent was found to give adequate resolution of galactose and mannose in all cases. The single development time was 30-40 minutes for the thin layer and slightly over one hour for the filter paper techniques. The plates or the paper were sprayed with either p-anisidine-phthalate (Reagent I) or 3,5-dinitrosalicylate (Reagent II) as described by Jeanes, et al (1951). The presence of both mannose and galactose in fibrinogen and fibrin were confirmed (Plate #1). The same amount of galactose and mannose appear in both fibrinogen and fibrin. A more detailed study of the rates of galactose and mannose released during graded acid hydrolysis is being conducted.

To test for sialic acids, 100-150 mgm. of fibrinogen were heated in a glass marble-covered tube with 10 ml. of 0.01 N H₂SO₄ for one hour at 90°C. as recommended by Svennerholm (1956). Three of these hydrolysates were combined and treated in the manner described for the isolation of hexoses. The paper partition chromatography of the sialic acids was conducted in the manner described by Svennerholm. The sialic acid reagents which were found the most effective were (1) resorcinol and (2) Ehrlich's

Plate 1 - The undimensional multiple run (3) chromatographic separation of mannose, galactose, and glucosamine on Whatman #1 (5 x 10 cm.) paper with n-butanol:pyridine:water (6:4:3) developer. Chromatogram A. represents 10 ugms. each of mannose, galactose and glucosamine and the mixture of the three standards. Chromatogram B represents a mixture of 5 ~~ugms~~ each of mannose and galactose compared with the acid digest of fibrin treated as described. Chromatogram C represents a mixture of 5 mgms each of galactose and mannose compared with the acid digest of fibrinogen.

**CHROMATOGRAPHIC IDENTIFICATION OF
MANNOSE AND GALACTOSE IN
FIBRINOGEN AND FIBRIN DIGESTS**



reagents (Gottschalk, 1960). A comparison of human α_1 -glycoprotein similarly treated was made and the results are shown in Plate #2. The most suitable reference standards would have been the N-acetyl and NO-diacetyl neuraminic acids derived from human submaxillary glands. The preparation of neuraminic acids from human submaxillary glands has not as yet been described, however, the method of Blix (1936) described for the ovine (sheep, N-acetyl neuraminic acid) types of sialic acids would be most appropriate to attempt such an isolation.

c) Some kinetic considerations regarding the preparation of fibrinogen-hydrazide and its physico-chemical behavior.

Samples of 150-200 mgm. of human fibrinogen were treated in the same manner as described for human albumin. The samples were individually treated with 1 M hydrazine, pH 9.0 for various periods of time (15 to 240 minutes). The elution of the human fibrinogen-hydrazide from the previously described Sephadex column is shown in Figure 1. The mole ratios of heat-labile hydrazine to protein were from 0.54 to 3.44, depending on the time of treatment. The near maximum amount of hydrazine bound to fibrinogen (3.0 ratio) was found to occur in somewhat less than 90 minutes and did not substantially increase upon prolonged incubations (4 hours). The results are illustrated in Figure #2.

The following experiments were done in order to determine the optimal pH conditions for the reaction between

CHROMATOGRAPHIC IDENTIFICATION OF
SIALIC ACID IN FIBRINOGEN DIGESTS

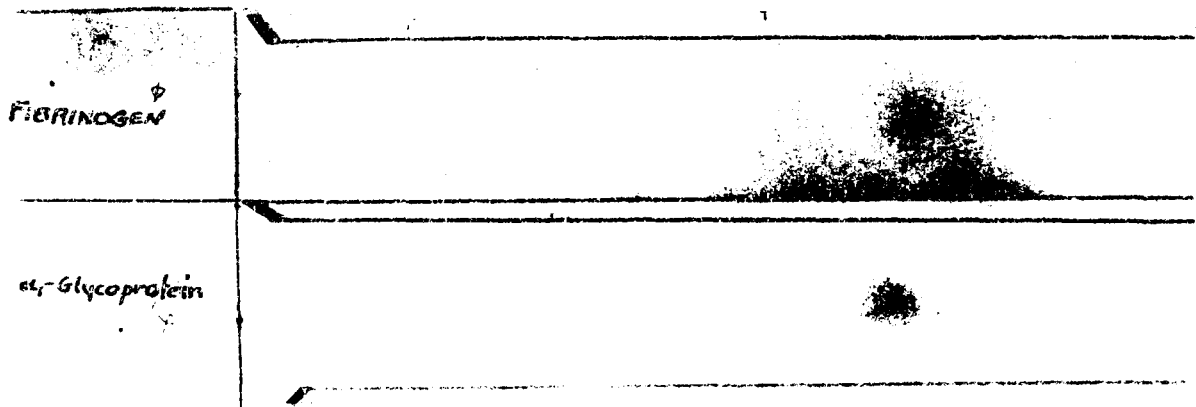


Plate 2. - The chromatographic comparison of the sialic acids derived from human α_1 -glycoprotein and human fibrinogen was developed with n-butanol:n-propanol:0.1 N hydrochloric acid (1:2:1) and identified with resorcinol reagent. The RF values for the derived sialic acids were 0.51 and 0.53 for α_1 -glycoprotein and fibrinogen, respectively.

*KINETICS OF THE REACTION OF
FIBRINOGEN WITH 1M HYDRAZINE*

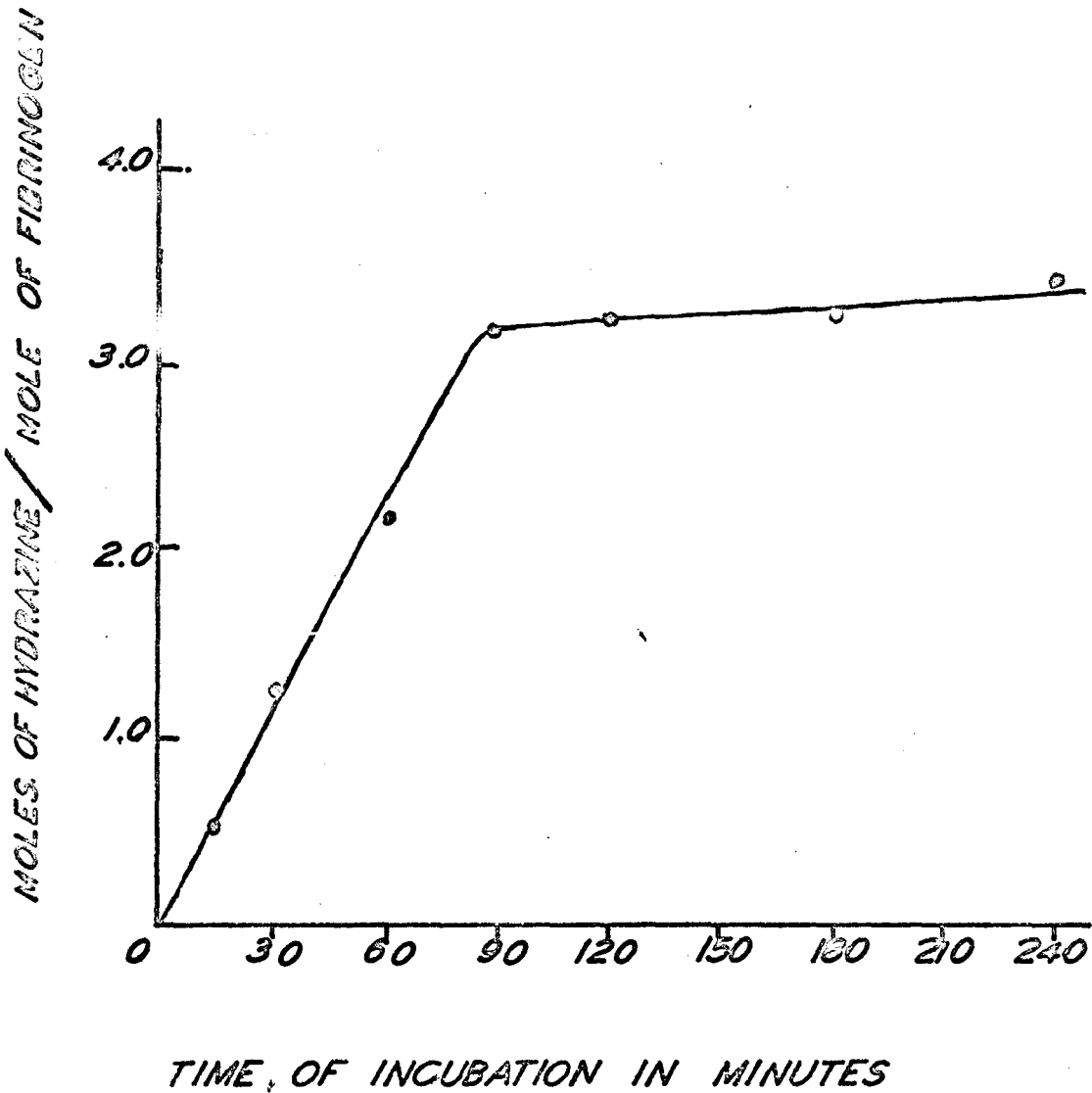


Figure 2. - The reaction of 1 Molar buffered (pH 9.0) hydrazine and human fibrinogen at 37°C. and the moles of "heat-labile" hydrazine formed per mole of fibrinogen during the reaction were determined as described in the text.

human fibrinogen and hydrazine: Human fibrinogen samples of 150-200 mgm. size were then treated with 1 M hydrazine at various hydrogen ion concentrations (pH 6.0 to 10.0) for 90 minutes. The pH measurements were made at 23°C. with a Beckman Model G pH meter using an E-2 glass electrode recommended for alkaline solutions. The Figure #3 indicates the pH dependence of the hydrazine reaction with human fibrinogen, and likewise suggests that for the conditions used in this study, that the maximum reactivity for hydrazine with fibrinogen is attained at pH's above 9 (hydrazine $pK_a = 8.5 @ 24^{\circ}C$).

The effects of varying molar concentrations of hydrazine and reaction times at different pH values, and at different temperatures were not investigated.

A number of control experiments were made in order to establish that "heat-labile" hydrazine (fibrinogen-hydrazide) found in treated fibrinogen was not present in untreated or experimentally modified fibrinogens. First, a 163 mgm. sample of fibrinogen was brought to pH 9.0 with 0.1 M sodium hydroxide in a final volume of 10 ml., and carried through the prescribed procedure for labile hydrazine detection. Second, a 144 mgm. sample of fibrinogen was treated with 1 M hydroxylamine, pH 9.0, and similarly examined. Third, a sample (0.89 mgm.) of untreated fibrinogen dissolved in 2 ml. of 1% MCA was heated with the Ehrlich's reagent as described.

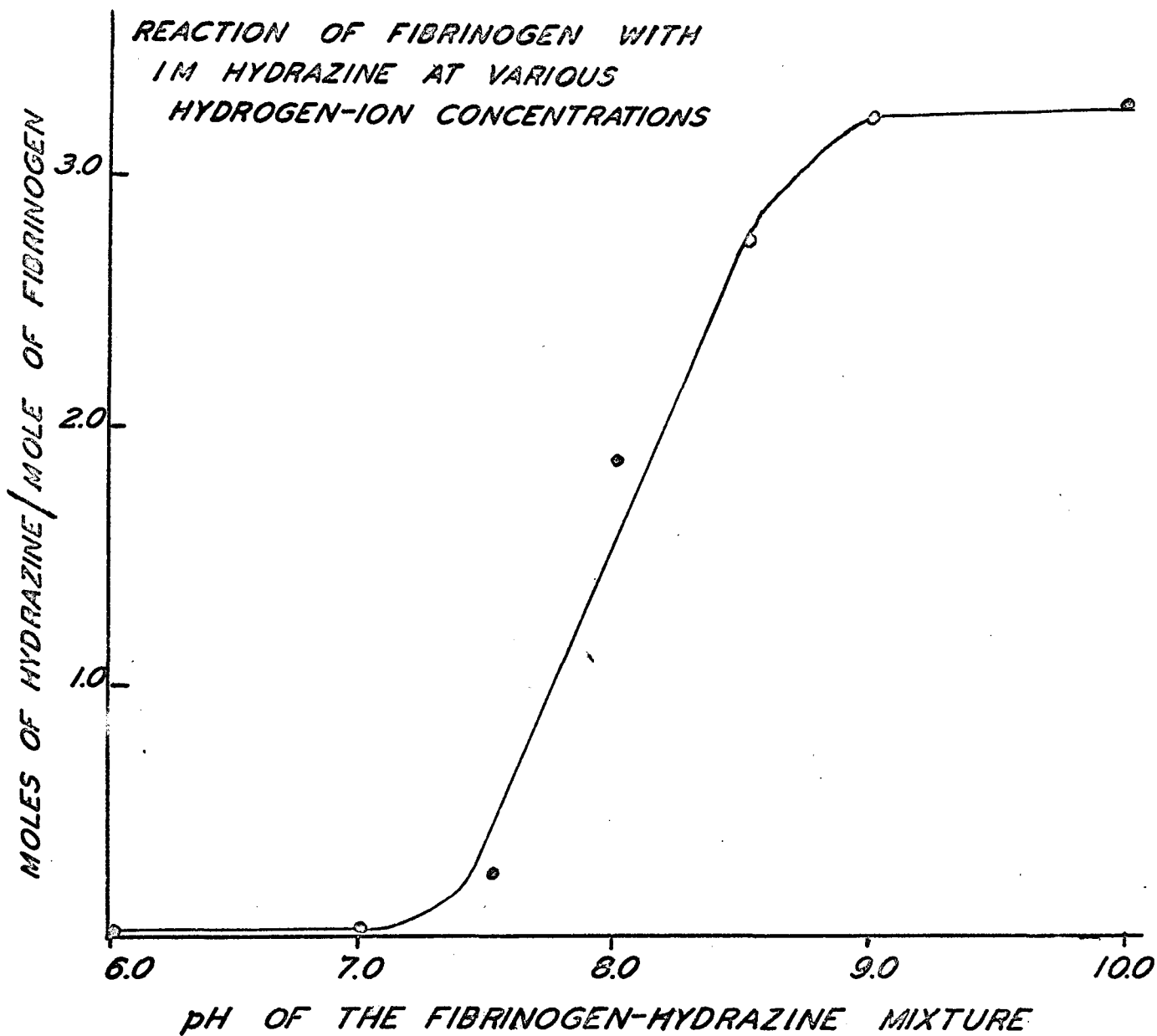


Figure 3. - The pH dependence of the reaction between human fibrinogen and hydrazine. The mixtures were each incubated at 37°C. for 90 minutes and the moles of "heat-labile" hydrazine formed per mole of fibrinogen were determined as described in the text.

In none of these control studies was an absorption maximum at 460 mu revealed upon heating with Ehrlich's reagent. The maximum absorption wavelength for free hydrazine reacted with Ehrlich's reagent is 460 mu. The failure of the controlled experiments to reveal an increase of absorbance after heat treatment was taken to mean that no heat-labile substances interfering with the hydrazine treatment were present in the controls. The recordings of absorbances at all wavelengths between 700 and 400 mu were made with a Beckman DB in 1 cm. cells. The recordings were made for the hydrazine-treated fibrinogen and the fibrinogen controls, which were both treated with Ehrlich's reagent (unheated and heated). Chart #1 represents a comparison of the hydroxylamine-treated sample (line 1) with the hydrazine-treated sample (line 2). Both samples contained the same amounts of protein (1.48 mgm.) by Kjeldahl nitrogen analyses, and both were heated with Ehrlich's reagent as described. The dual beam spectrophotometer was set at zero absorbance with the unheated Ehrlich's reagent-protein samples as the reference blanks.

The Chart #2 is a recording of the hydroxylamine-treated sample (line 1 - Chart #1) being used as the reference blank and recording the hydrazine-treated sample as the unknown. This comparison indicates the "heat-labile" hydrazine as line 1. The line 2 of the Chart #2 represents a comparison of both samples treated with Ehrlich's reagent but unheated. The fact that a

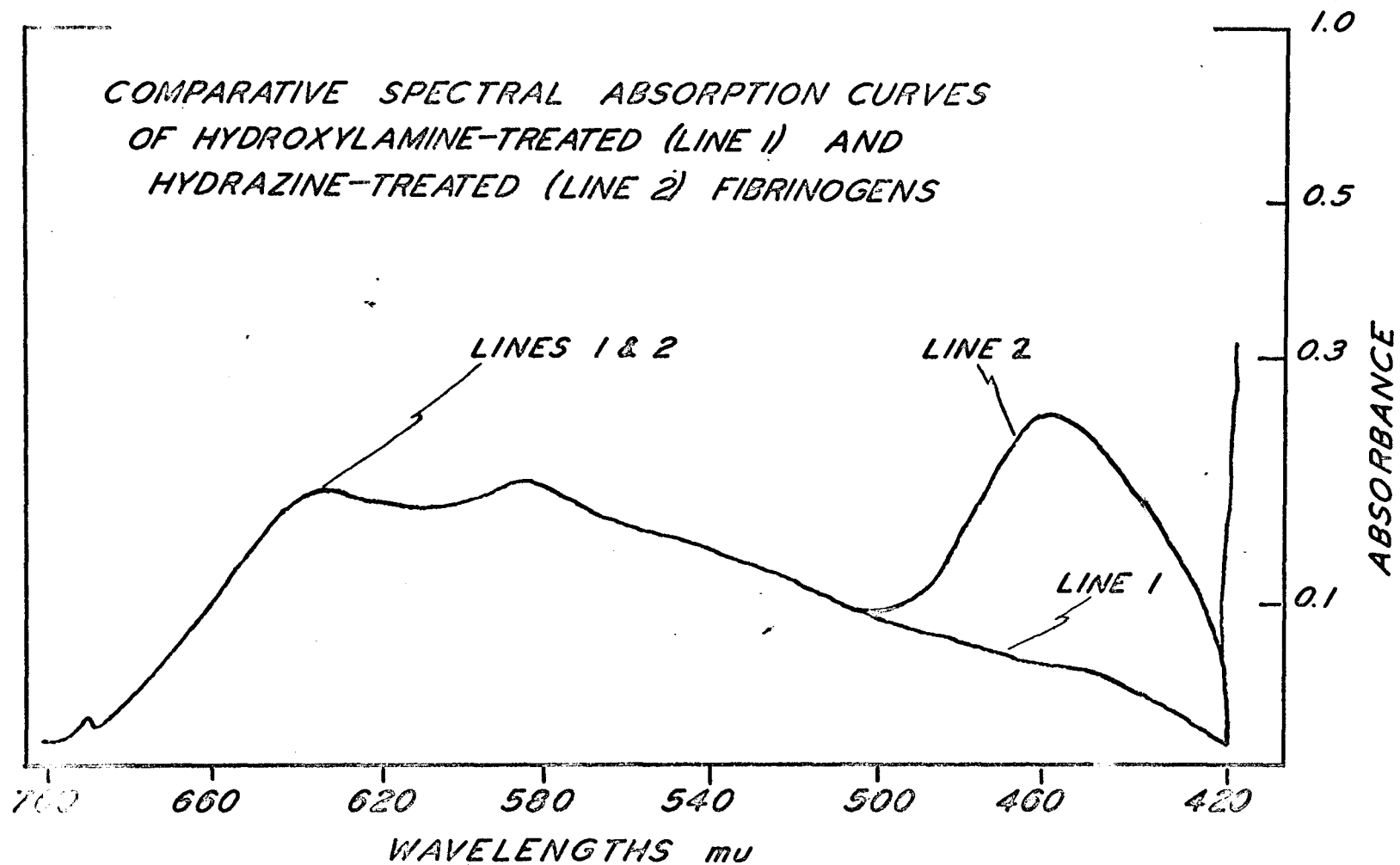


Chart 1 - Equal amounts (1.47 mgms.) of hydrazine-treated and hydroxylamine-treated (control) human fibrinogens were treated with Ehrlich's reagent as described in the text and the spectral absorption curves were obtained on a Beckman DB recording instrument.

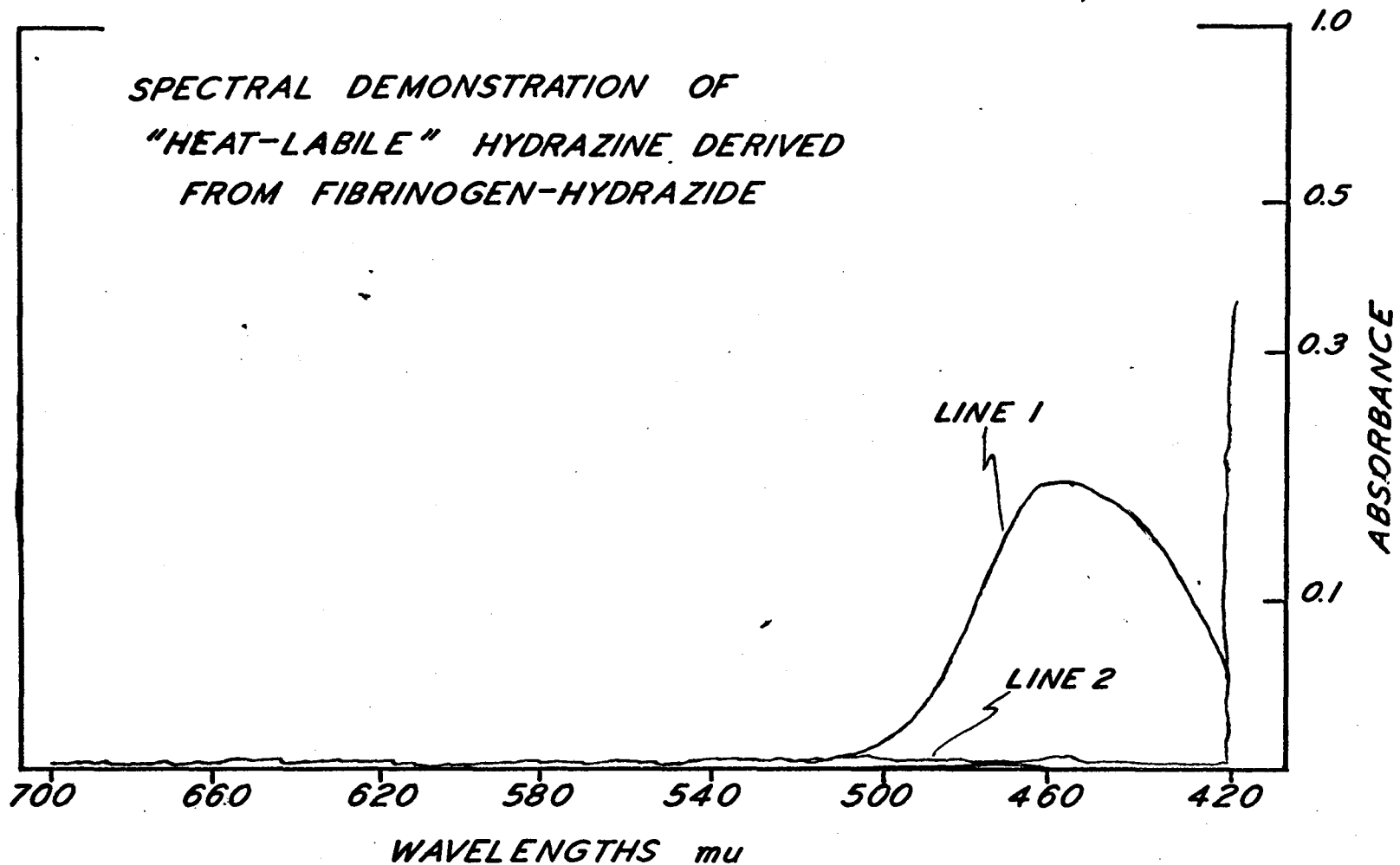


Chart 2. - A spectral comparison of 4.5×10^{-3} micro moles of hydrazine-treated fibrinogen heated (30 minutes at $80^{\circ}\text{C}.$) with Ehrlich's reagent (line 1) and mixed with Ehrlich's reagent but not heated (line 2). The reference cell contained the hydroxylamine-treated fibrinogen (control) which was similarly treated with Ehrlich's reagent.

very small amount (less than 0.001 μM) of free hydrazine (line 2 - Chart #2) appears to be present is probably due to the heating of the cuvette contents occurring during the recording period.

Further evidence regarding the existence of fibrinogen-hydrazine was obtained when equal amounts (17 mgm. each) of the alkaline-treated fibrinogen and the fibrinogen-hydrazide were reacted with an alkaline-copper tartrate solution and examined for copper reduction by the neocuproine method described by Brown (1960). The fibrinogen-hydrazide was able to reduce about twice as much copper as the control fibrinogen sample. The neocuproine measurements of alkaline copper reduction due to hydrazine are sensitive in the ranges of 0.1 to 1.0 μMoles of hydrazine in a total volume of 10 milliliters.

The average total hexose content of three hydrazine-treated samples was 1.5%.

It appears that under the conditions described in these experiments that 3 moles of hydrazine have reacted with 1 mole of fibrinogen to form a "fibrinogen hydrazide". The absorption maxima at 630 and 580 μu wavelengths for fibrinogen and other proteins heated with Ehrlich's reagent will be presented in Part c of this Section.

Some physico-chemical studies were made on the fibrinogen hydrazide preparation which was eluted from the column with 0.15 M acetate buffer, pH 5.5 instead of 1% MCA. The

fibrinogen-hydrazide eluted from the column was not clottable. In a control experiment the fibrinogen brought to a pH 9.0 with dilute sodium hydroxide, incubated for 90 minutes at 37°C., and then eluted from the column with the acetate buffer was found to be 84% clottable. It was concluded that the loss of clottability on treatment with buffered hydrazine was not due to the denaturing effects of the prolonged alkali treatments which were used in these experiments.

The electrophoresis of the fibrino-hydrazide and the untreated fibrinogen was performed with the power supply and paper electrophoresis cell described by the author (1953). The electrophoresis was at 150 volts for 5 hours, at +5°C. using Whatman 3MM paper with barbital buffer, pH 8.65, and ionic strength 0.05. Paper electrophoresis of these fibrinogen preparations was also performed with acetate buffer, pH 5.5, ionic strength 0.05, at 150 volts for 7 hours at +5°C. The proteins were stained with light green SF or ninhydrin spray. At the pH 8.65, the hydrazine-treated fibrinogen had a slightly slower mobility toward the anode than did the untreated fibrinogen. At the pH 5.5, neither of the fibrinogen preparations demonstrated a mobility, since this was the isoelectric value (5.5) for fibrinogen (Mihalyi, 1950).

The sedimentation characteristics of the hydrazine-treated fibrinogen and the fibrinogen (fraction I₄)

preparations were observed and compared using a double sector cell in the Spinco Model E analytical ultracentrifuge.* The treated fibrinogen was prepared as previously described, but eluted from a 0.3 M NaCl equilibrated Sephadex G-50 column with 0.3 M NaCl solution. The eluates containing the treated fibrinogen were concentrated by dialysis over-night at +5°C. against a polyethylene glycol compound (Carbowax 20-M). The fibrinogen (fraction I₄) and the treated fibrinogen were both made to a 1% solution with 0.3 M NaCl as the diluent. Both preparations were sedimented in the ultracentrifuge at 20°C. for 1 hour and 20 minutes after the full rotor speed of 52,640 rev./min. was obtained, and photographs taken at the Bar angle 55° (Plate #3). The untreated fibrinogen sedimented as a homogenous peak throughout the run. The treated fibrinogen appears to sediment at the same rate, but exhibits a peculiar and unexplained aggregation of molecules.

A preparation of human fibrin was not readily soluble in 1 M hydrazine, pH 9.0, therefore, approximately 150 mgm. of fibrin was dissolved in 5 ml. of 2 M NaBr and then 5 ml. of 2 M hydrazine, pH 9.0, was added. The incubation and removal of the free hydrazine was performed as in the case of fibrinogen.

* The ultracentrifuge runs were done through the courtesy of Dr. F. Rothstein, Retina Foundation, Boston, Mass.

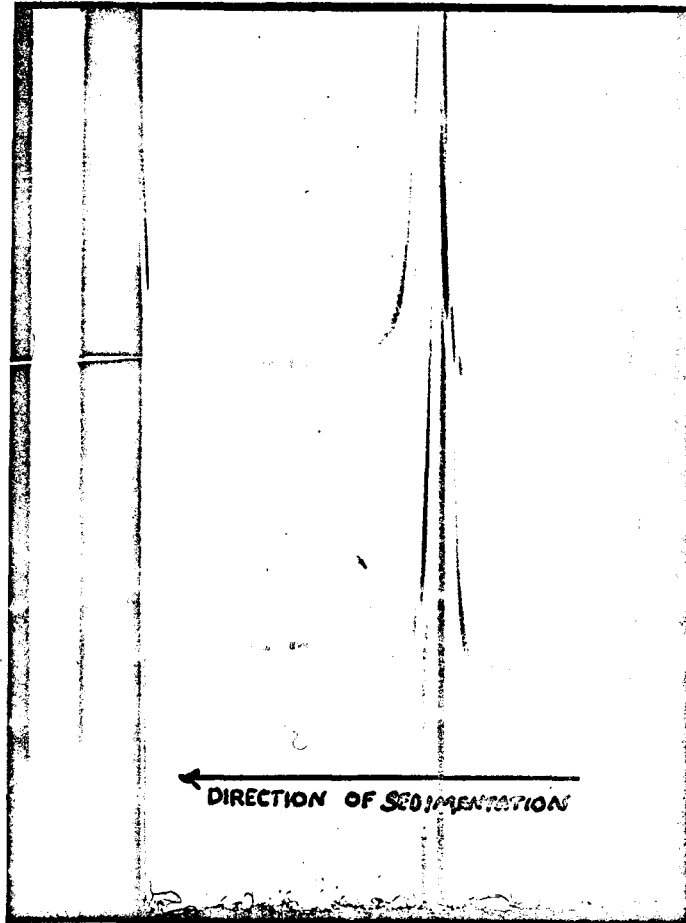


Plate 3. - The sedimenting characteristics of hydrazine-treated fibrinogen (upper) and untreated fibrinogen (lower). A standard double sector cell was employed; rotor speed: 52,640 rev./min.; Bar angle 55°; time of picture 80 minutes.

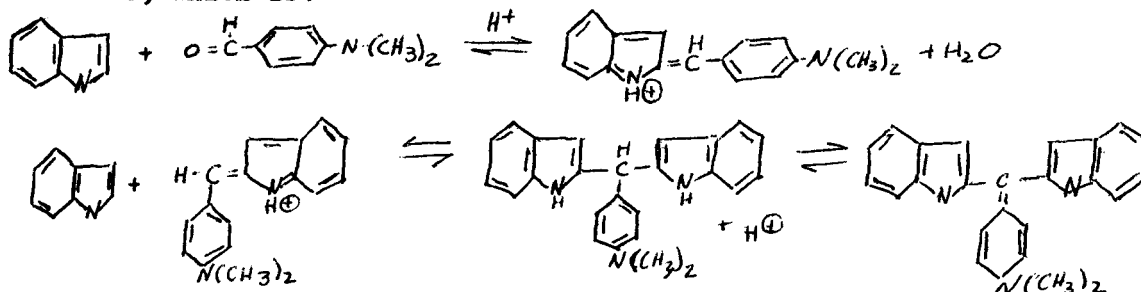
The mole ration of heat-labile hydrazine to fibrin was found to be 2:95. This ratio indicates a reduction of 0.25 in the mole ratio which was found for fibrinogen; however, the kinetic studies and controls for the fibrin experiments were not completed at the time of this writing.

d) The possible interference of tryptophan and the spectrometric characteristics of various proteins treated with Ehrlich's reagent.

The proteins used in this investigation contain variable amounts of tryptophan, and the Ehrlich's reagent has been used to determine this amino acid in several proteins. Therefore some studies were conducted to determine the extent of interference in the sialic acid determination caused by the presence of tryptophan residues in fibrinogen.

There are a variety of p-dimethylaminobenzaldehyde: ethanol:hydrochloric acid mixtures described by Dalgleish (1955) which are all referred to as "Ehrlich's Reagent" (ER). The Ehrlich's condensation-reaction is also variable with respect to temperature, time and other conditions. The Ehrlich's reaction has been used to detect indoles, pyrroles, proteins, amino acids, sialic acids, sugar and hexosamines condensed with acetyl-acetone. The reaction of ER with tryptophan is said to produce a red to green coloration, and the chromogen absorbs at wavelengths between 600 and 520 mu. The condensation-reaction of Ehrlich's

reagent with tryptophan is said to be similar to that given for indoles, which is:



A composite of the impressions given by Fiegl (1956), Mauzerall and Granick (1956).

The Ehrlich's reagent and its use in these experiments was described in Section IIIa. When hydrazine sulfate, l-tryptophan and the heat-labile hydrazine derived from fibrinogen-hydrazide were each subjected to the Ehrlich's reaction, there were observed three very similar spectral responses (Chart #3). All of these compounds indicated a maximal absorption at the wavelengths of 460 mu. However, the reaction of hydrazine (0.025 uM) with ER was found to be about a thousandfold more sensitive than that with l-tryptophan (20.0 uM). Furthermore, the reaction with hydrazine was complete at room temperature (23°C.) in less than 5 minutes and did not increase the color density upon further heating (80°C). The addition of 2 mgm. (air-dry weight) each of human albumin or commercial gelatin to 10 uM of l-tryptophan did not show an increase in absorbance at 460 mu with ER which was greater than

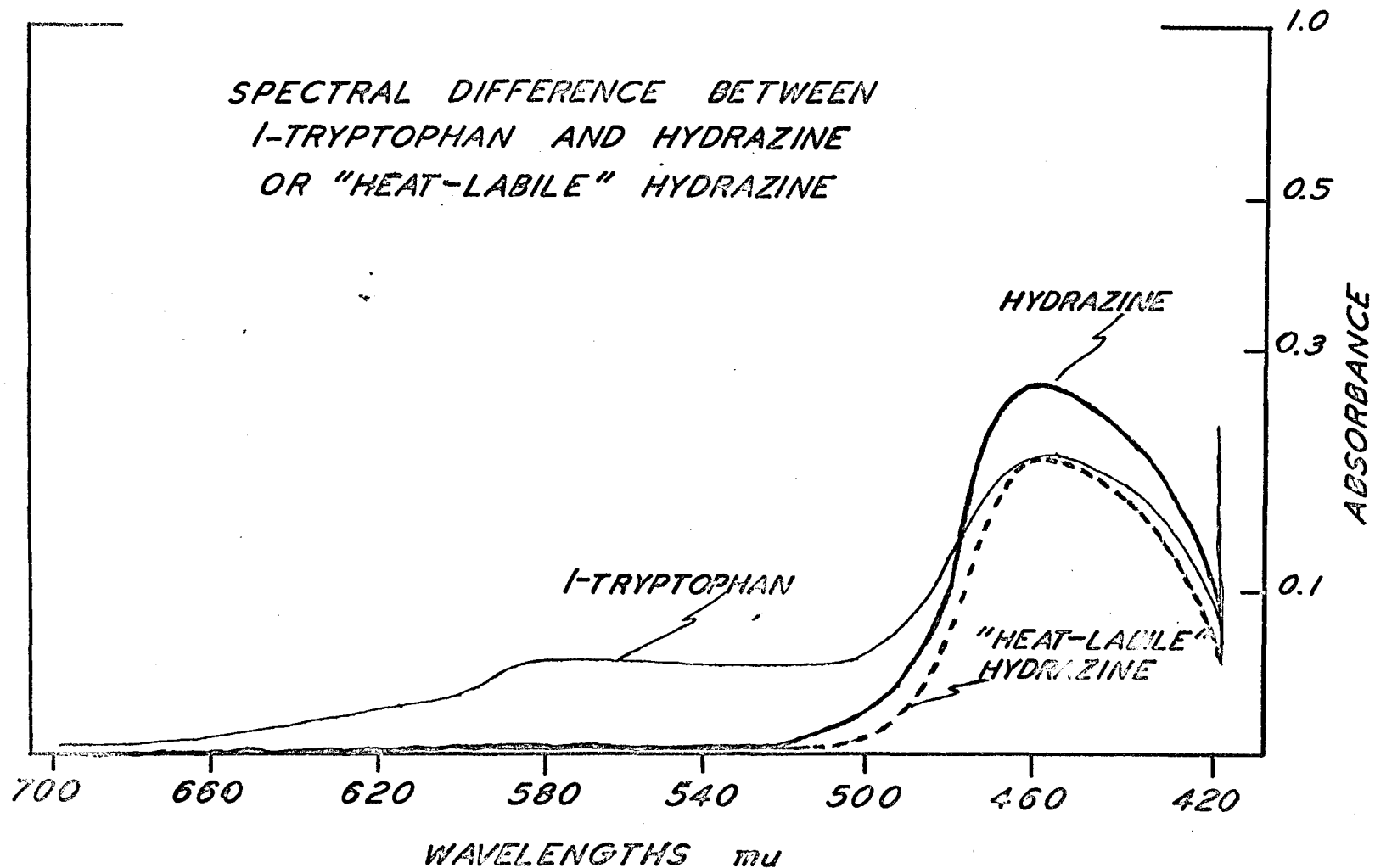


Chart 3. - Hydrazine sulfate (0.025 μ M), l-tryptophan (20 μ M), and human fibrinogen-hydrazide (1.76 mgm.) were each treated with Ehrlich's reagent in the manner described under Section IIIa. The protein nitrogen equivalent of untreated fibrinogen was used as the reference blank for the fibrinogen-hydrazide ("heat-labile" hydrazine). In the other two cases a solution of 1% monochloroacetic acid treated with Ehrlich's reagent was used as the reference blank. The spectral charts were obtained on a Beckman DB recording instrument using 1 cm. cuvettes.

could be accounted for by the proteins and l-tryptophan, separately tested.

It was concluded that the presence of proteins in the Ehrlich's reaction could not account for the increased 460 m μ absorbance which was observed when the fibrinogen-hydrazide preparations were heated to cause the liberation of free hydrazine.

The possible disturbing influence of l-tryptophan upon the method used for the determination of hydrazine led to an investigation of other compounds which were: l-tyrosine (40 μ M), l-proline (40 μ M), glycine (50 μ M), and 10 μ M each of di-, tri-, and tetraglycine, D-mannose, D-galactose, D-glucose, D-glucosamine, N-acetyl-D-glucosamine, and N-acetyl neuraminic acid (sialic acid). None of these compounds when heated with the Ehrlich's reagent would demonstrate any marked spectral characteristics at 460 m μ . All of the amino acids (except proline), the peptides, and the glucosamines revealed a sharp spike-like absorption at 420 m μ . This spectral behavior was regarded as the condensation products of ER and the various primary amines. The N-acetyl neuraminic acid demonstrated a peak absorption of 565 m μ , which was consistent with that demonstrated by Werner and Odino (1952) for the various glycoproteins containing sialic acids.

The unique spectral curve-changes at 630 and 580 m μ

for the plasma proteins treated with ER prompted the exploration of other proteins. In addition to the plasma proteins used in the hydrazine binding experiments, the following proteins were reacted with ER: twice crystallized pepsin (Worthington Bio. Corp. lot #642), chymotrypsin (Ref. #311-A89), beef zinc-insulin (E. Lilly Co. lot #693502), twice crystallized trypsin salt-free (Worthington Bio. Corp. lot #823-24), twice crystallized lysozyme derived from egg white (Mann Res. Lab. lot #G-2056), and crystallized ovalbumin (Nat. Bio. Lab. lot #G9855). The protein nitrogen conversion factors and (molecular weights) were taken as: chymotrypsin 6.17 (25,000); trypsin 6.25 (23,800); lysozyme 5.38 (15,000) and insulin at pH 2.0 (6,000). No corrections were made for zinc content since it has been found to vary considerably in the commercial preparations. The air-dry weight of insulin was taken as the protein content in these calculations. The protein concentrations of the other samples were determined by Kjeldahl analyses.

The resulting spectra of the various proteins which were heated with ER as previously described are shown in Chart #4. Both pepsin and ovalbumin were insoluble in either the alcohol or the acid contained in the ER, or in the 1% monochloroacetic acid solution. The last two proteins did show a turbid blue coloration. All of the other proteins demonstrated varying degrees of clear blue-green-yellow coloration. The

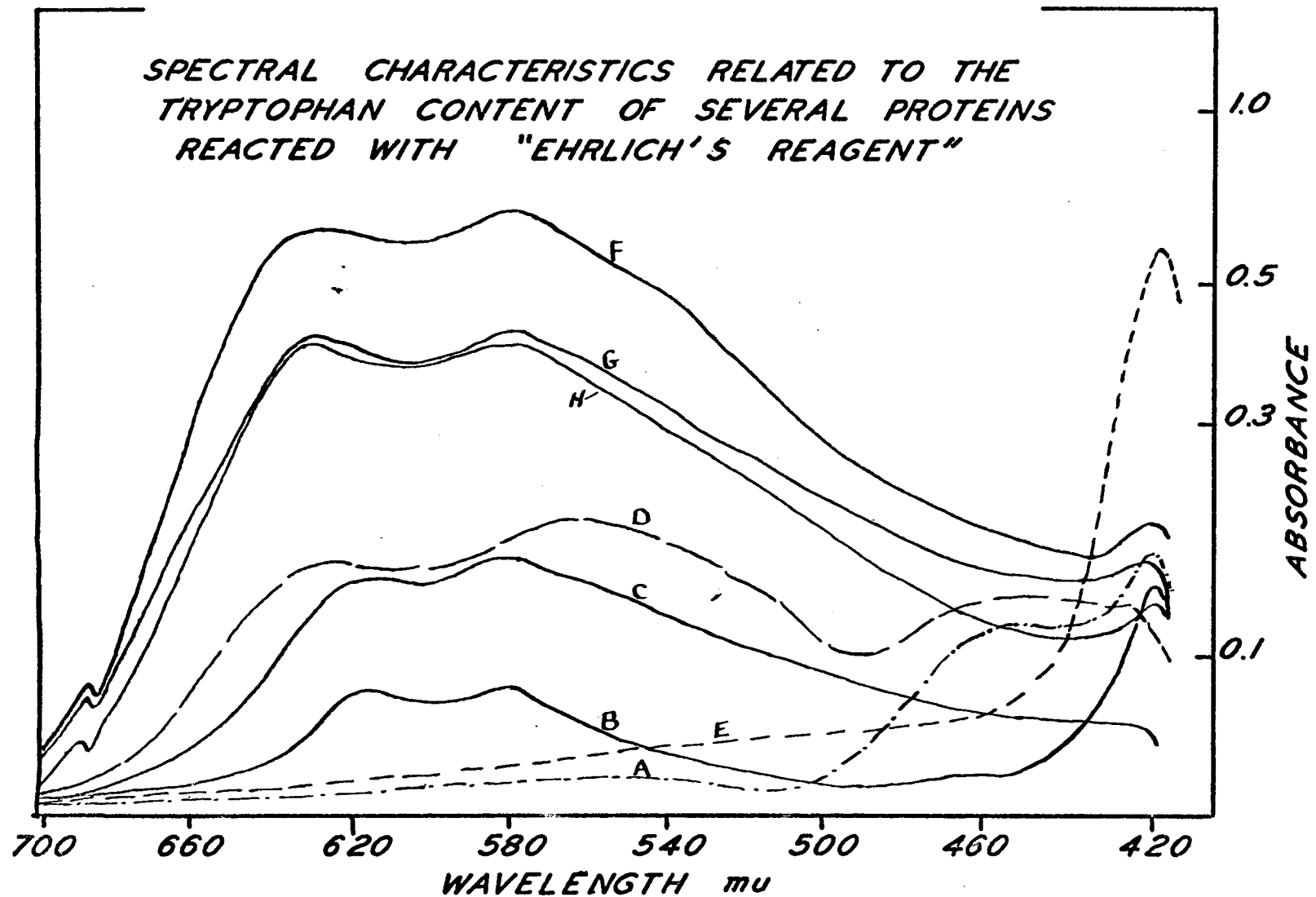


Chart 4. The following proteins were treated with "Ehrlich's reagent" as described in text:

- (A) Human Albumin (B) Bovine Albumin (C) Human Fibrinogen (D) Human α_1 -glycoprotein
(E) Zn-insulin (F) Chymotrypsin (G) Trypsin

X₁-glycoprotein contained a slight shade of pink-red, undoubtedly due to its sialic acids content. The relationship of the stable blue coloration to the tryptophan contents of the proteins were first described by Voisenet (1905) and Rhode (1905). The relationship of absorbancy at 600 mu and the mole ratios of tryptophan in proteins are illustrated in Table II. Casein has been used in the past as a reference standard for the determination of tryptophan in proteins by the Ehrlich's reaction. The results obtained with crystallized lysozyme in these experiments would suggest that it may serve as an excellent reference standard for the determination of peptide-bound tryptophan in proteins.

The conditions used in these experiments suggests that the number of "buried" tryptophyl groups or residues, are directly related to the absorbance at 600 mu wavelength. The unusual 460 mu absorption behavior of both human albumin and

X₁-glycoprotein when heated with Ehrlich's reagent could suggest tryptophan bound groups or tryptophanyl groups "exposed" in the terminal peptide chains of proteins. Human albumin is known to bind one mole of tryptophan per mole (McMenamy and Oncley, 1958). No information is available concerning "exposed" or tryptophan binding to X₁-glycoprotein. There are no reports concerning the possibility that "exposed" or "bound" tryptophyl groups are more sensitive to the Ehrlich's reagent than free tryptophan. In view of the sensitivity of the "buried" tryptophyl groups to the ER, it would not be unusual to

Table II

THE SPECTRAL CHARACTERISTICS OF VARIOUS PROTEINS TREATED WITH
"EHRlich'S REAGENT" AND THEIR RELATIONSHIP TO THE TRYPTOPHAN
RESIDUES PER MOLE OF PROTEIN

#	Protein	Protein Concentration uM x 10 ²	Absorbance @600 mu x 10 ²	Absorbance ratio Mole Protein	Tryptophan* residues/ mole protein
A	Human Albumin	6.2	1.5	0.2	0.63 (a)
B	Bovine Albumin	4.0	6.5	1.6	2.3 (a)
C	Human Fibrinogen	0.6	16.0	26.6	52.8 (a)
D	α ₁ -glycoprotein (Human)	4.6	15.5	3.4	3.8 (b)
E	Zn-insulin	458.0**	2.0	0.0	0.0 (a)
F	Lysozyme	14.5	58.0	4.0	7.7 (c)
G	Chymotrypsin	11.8	38.2	3.2	7.0 (d)
H	Trypsin	13.5	36.2	2.7	X (d)

* The tryptophan residues/mole of protein were calculated or obtained from following references: (a) Tristram (1953); (b) K.Schmid, et al (1962); (c) Anfinsen and Redfield (1956); (d) Desnuelle (1960).

** No correction was made for Zn content, and calculations are based upon air-dry wt. basis.

find that the "exposed" tryptophyl groups were somewhat more sensitive to ER than free tryptophan. However, tryptophan interference in the measurements obtained for the "heat-labile" protein hydrazide must be quantitatively small, less than 0.5 per cent.

III. The release of carbohydrates and peptide nitrogen during the conversion of fibrinogen to fibrin

a) An introductory experiment

An appropriate introduction which suggested that the carbohydrate changes did occur during the conversion of human fibrinogen to fibrin was indicated in the following simple experiments.

Paired samples of either oxalated or untreated whole blood were collected in approximately equal volumes (10 ml.) from eleven different hospitalized patients. All the samples were allowed to remain at room temperature for approximately one hour, and then centrifuged for 20 minutes. The plasma or sera were drawn off and again centrifuged for 20 minutes to insure the complete removal of erythrocytes. The dialyzable reducing substances in the samples were measured by the alkaline ferricyanide method as adapted to an Auto-analyzer (Technicon Co.). The reducing substances expressed as glucose ranged from 208 to 72 mgm. per 100 ml. of plasma or serum. The sera values were from 1 to 4 mgm.% higher than the plasma values, with the average

increase of 2.3 mgm.%. The "t" test for small samples was done, and a t_{10} value of 5.5 was found, which indicated that the differences were statistically significant at the probability level of less than 0.001. Three samples each of oxalated plasma were treated with a small crystal of anhydrous calcium chloride, and incubated at 37°C. for 1½ hours, along with the untreated plasma. The plasma which was allowed to clot was 1 to 2 mgm.% higher than the untreated plasma.

Obviously, blood sugars have been done in the routine clinical laboratory for over forty years, however, the difference of 1 or 2 mgm.% in a blood sugar would have little or no clinical significance. Furthermore, the measurement of dialyzable reducing substances was only recently introduced into the clinical laboratories (1957-8), and the paired plasma-sera analyses of dialyzable reducing substances has not been hitherto reported.

b) Method of assay

The final simple techniques which are presented here were developed after many partially successful or otherwise lengthy experimental approaches were discarded. Although the reagents used in these experiments are common to the biochemist, they were altered so much in their concentrations, volumes and applications, that a detailed presentation was inevitable. With the proper micro-instrumentation the method presented here could be applied to measure the peptide nitrogen changes in as little

as 2 mgm./ml. of fibrinogen converted into fibrin.

The purified human fibrinogen (fraction I₄) and the human thrombin was obtained as previously described. Approximately 11 grams of the frozen fibrinogen was dissolved at room temperature (ca. 23°C.) in 50 ml. of 0.30 M NaCl. The fibrinogen was readily soluble within 20 minutes when dissolved with the aid of a magnetic mixer. The fibrinogen solution was then diluted to 100 ml. volume with a phosphate buffer, pH 6.4, ionic strength 0.1 (this buffer contains 0.003 M Na₂HPO₄; 0.012 M KH₂PO₄, and 0.075 M NaCl). Triplicate Kjeldahl protein nitrogen analyses revealed that the solution contained 32 mgm. of fibrinogen per ml. Two ml. each of this fibrinogen solution were placed into 25 x 100 mm. round bottom tubes (except in those experiments relating to varying fibrinogen concentrations). A mixture of 0.3 M NaCl; phosphate buffer (1:1) was added to make the final volume equal to 4 ml. when the thrombin solution was added. The tubes were pre-incubated for 10 minutes at 37°C. before the addition of the thrombin solution.

The volume of the thrombin solution was usually 0.5 ml. (2.70 NIH units) except in those cases where the effects of varying concentrations of thrombin were studied. The thrombin solutions were prepared shortly before each experiment by diluting the stock thrombin (270 NIH units per ml.) with the NaCl;phosphate buffer mixture. Immediately after the addition of the thrombin a

stopwatch and the time clock were started and the clotting times were noted. Soon after the addition of the thrombin, glass rods were placed into each of the incubation tubes. About one minute before the designated periods of time which were 10, 30, 60, 90, 120, 180, and 240 minutes, the glass rods were used to collect the clots and these clots were squeezed against the sides of the tubes in order to express as much solution from the fibrin clots as was physically possible. The removed clots were analyzed for fibrin content by the alkaline-urea U.V. absorption method.

The unclotted fibrinogen and thrombin were precipitated by the addition of 1.5 ml. each of 7.5% (w/v) zinc sulfate ($\text{ZnSO}_4 \cdot 5\text{H}_2\text{O}$) solution and a barium hydroxide solution. The barium hydroxide solution was prepared from a saturated solution which was diluted until it would exactly neutralize an equal volume of the 7.5% zinc sulfate solution at the equivalence point of phenolphthalein indicator.

The thrombin was added to the zero time tube after the precipitating reagents.

A control containing the fibrinogen solution without the addition of thrombin was incubated for 240 minutes and then treated with the precipitating reagents.

The mixed precipitates were allowed to remain in beakers of cracked ice for 10 minutes and then centrifuged at approximately 2500 rpm. for 10 minutes. The clear supernatants

were filtered through Whatman #1 filter papers.

Carbohydrate analyses were conducted by the anthrone- H_2SO_4 method using 3 ml. of each filtrate. In several cases the results were checked by the orcinol- H_2SO_4 method.

Peptide Nitrogen analysis reagents. The following modifications of the Lowry biuret-phenol reagents were necessary to obtain a final stable blue color: Reagent I contained 40 Gms. of anhydrous sodium carbonate and 1.0 Gms. of sodium potassium tartrate diluted to 1 liter with distilled water. Reagent II contained 0.5% (w/v) copper sulfate ($CuSO_4 \cdot 5H_2O$) in distilled water. Reagent III - the phenol color reagent was a 1:1 (v/v) mixture of the Folin-Ciocalteu reagent and distilled water.

The peptide nitrogen calibration curve was prepared using crystalline human albumin (4 to 16 μ gm. N per ml.) dissolved in the NaCl:phosphate buffer mixture as the reference standards.

Color Development - To one or two milliliters of the clear filtrate (10-30 μ g of peptide nitrogen) was added 3.5 ml. of reagent I and 0.5 ml. of Reagent II. When 1 ml. of filtrate was used, an additional 1 ml. of the phosphate-NaCl buffer mixture and water (1:1) was used to bring the volume to 2 milliliters. The mixture was incubated for 15 minutes at $37^\circ C$. and 1 ml. of the dilute phenol reagent was rapidly added, mixed, and further incubated at $37^\circ C$. for 15 minutes. The final blue color reached

a maximum intensity in 10 minutes and was stable for at least one-half hour. The colorimetric absorbances were made with a Coleman Model 14 at 660 m μ wavelength using 19 x 150 mm. cuvettes. The zero absorbance blank was 1 ml. each of the NaCl-phosphate buffer mixture and distilled water which was carried throughout the color development procedure.

Comments - The following types of filtrates could not be used with the phenol-biuret method for the peptide nitrogen analyses: TCA, phosphotungstic acid, or picric acid. The TCA filtrates resulted in a variable and unstable blue color development. Phosphotungstic acid precipitated all of the peptides, and picric acid could not be used because of its phenolic chemical behavior. The colorimetric Stein and Moore ninhydrin method was approximately 20 times less sensitive than the one described above.

Various other mMolar concentrations of zinc in the total volume of 7 ml. were tried. The concentrations of zinc between 30 and 60 mM in the volume of 7 ml. did not remove any of the human fibrinopeptides by precipitation. As the concentration of zinc was raised to 70 or 80 mM the second peptide was partially removed, but not the first. In the case of the bovine fibrinopeptides (A and B) the final concentration of 80 mM zinc did not precipitate either peptide. This behavior of the second human fibrinopeptide was the first indication that it may be

significantly different from that of bovine origin. The use of a $ZnSO_4$ - $Ba(OH)_2$ system for separating the unreacted fibrinogen and thrombin from the fibrinopeptides and carbohydrates offers at least three advantages: (1) the formed $BaSO_4$ provides an excellent absorbant for the removal of thrombin; (2) the filtrates are clear, consistent, and reproducible; (3) any protein contamination in the commercial heparin preparations are likewise removed.

c) Effects of increasing thrombin concentrations: Using the described assay system, the release of the peptides and carbohydrates were made with increasing amounts of human thrombin (0.005 to 2.0 NIH units/ml.) in the total volume of 4 ml. The fibrinogen concentration was constant at 16 mgm./ml. and the incubation time was 30 minutes in all cases studied. The results are shown in Fig. 4. Under these conditions a maximum release of peptide nitrogen and hexoses was obtained at 0.03 NIH units/ml. of human thrombin.

d) Effects of increasing fibrinogen concentrations: The thrombin concentration was constant at 0.34 NIH units/ml. and the fibrinogen concentration was varied between 8 and 32 mgm./ml. in a total volume of 4 ml. A slight decrease of the released peptide nitrogen was noted when the fibrinogen concentration increased. A similar decrease in the per cent clottability (43 to 36%) was also found. The results are shown in Fig. 5.

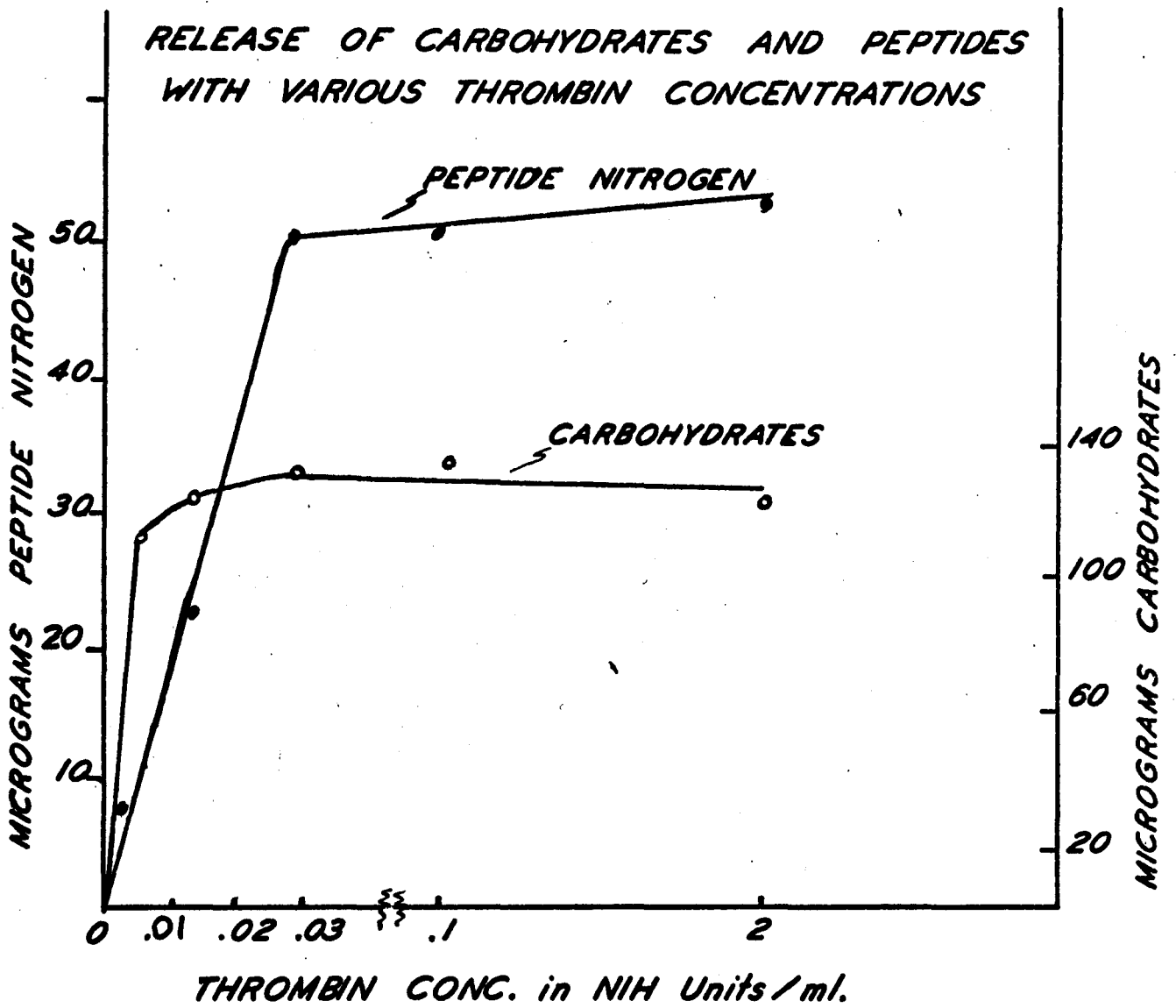


Figure 4. - ASSAY SYSTEM: 16 mgm. fibrinogen/ml. in 0.3 M NaCl:phosphate buffer (1:1), pH 6.4, u = 0.1. Total volume of 4 ml. incubated for 30 minutes at 37°C.

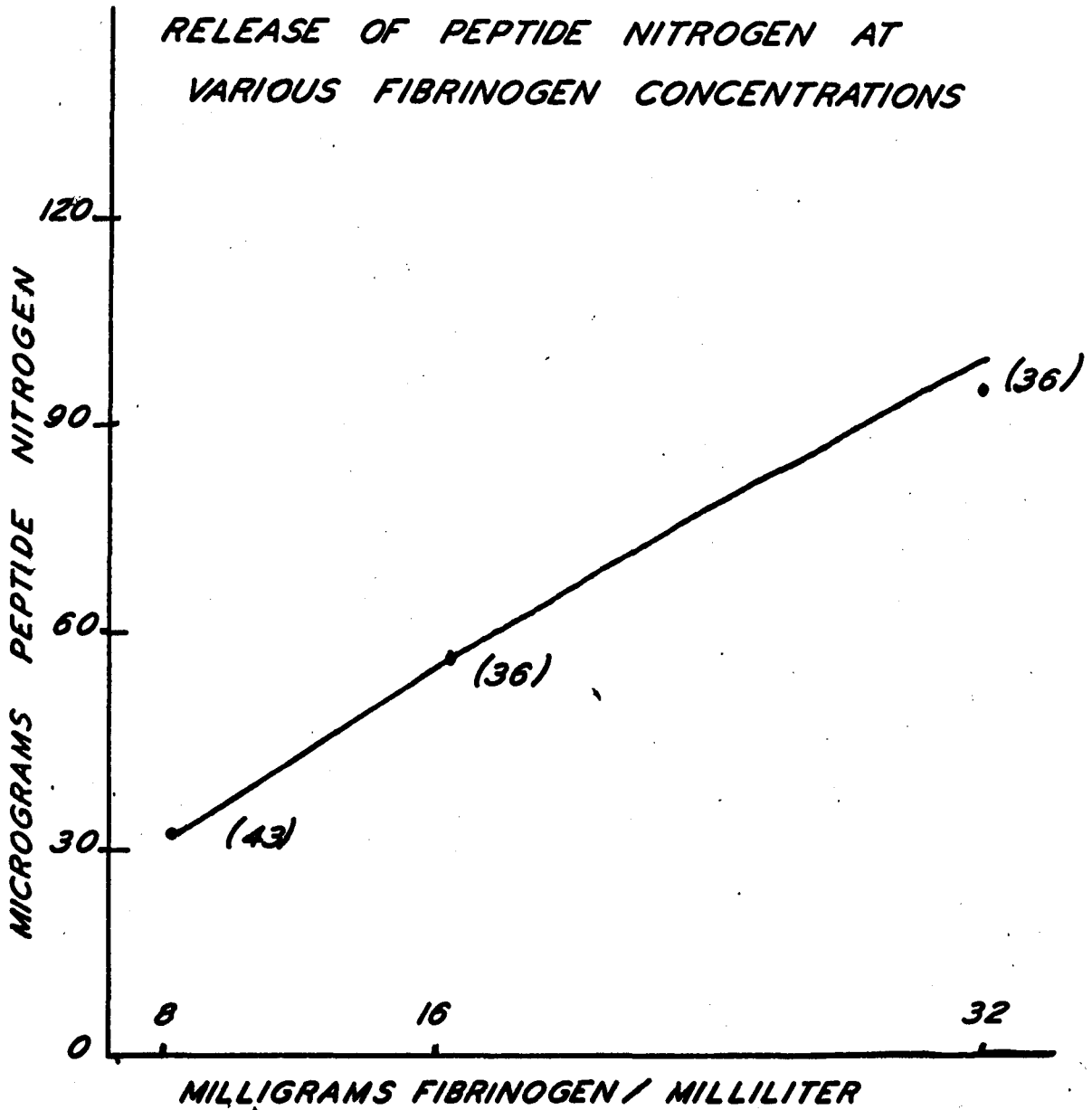


Figure 5. - ASSAY SYSTEM: 8, 16, or 32 mgm. of fibrinogen/ ml. in 0.3 M NaCl:phosphate buffer (1:1), pH 6.4, u = 0.1. The human thrombin concentration was 0.32 NIH Units/ml. Total volume of assay was 4.0 ml. Time of incubation at 37°C. was 30 minutes. () = per cent of fibrinogen clotted.

e) The release of peptides and carbohydrates with respect to time: The per cent of fibrinogen transformed into fibrin clot, the release of peptides and carbohydrates at various periods of time are presented in Fig. 6. The clotting time was $9\frac{1}{2}$ minutes as described by the first visual appearance of a fibrin clot. There was a direct relationship between the per cent of clot recovered and the first release of peptide nitrogen (designated as A). The second peptide (B) released was not related to the per cent of clot recovered. The second peptide released suggest that either another proteolytic enzyme system was auto-activated during the conversion of fibrinogen to fibrin or that a second proteolytic enzyme was present in the system which was specific for fibrin rather than fibrinogen. The fibrinogen control which was incubated to 240 minutes in the absence of thrombin was found to liberate 10 ug. of peptide nitrogen.

As mentioned previously, the fibrinogen used in these experiments may contain about ten per cent of the plasminogen which was present in the starting Cohn fraction I. This plasminogen can be autocatalytically converted into the active proteinase, plasmin, which will digest fibrin much more readily than fibrinogen. The second proteolytic type of enzyme which is present in the fibrinogen used in these experiments has been called 'fibrin-stabilizing factor', 'fibrinase' or L-L factor (Lorand, et al., 1959). The presence of fibrin-

RELEASE OF CARBOHYDRATES AND PEPTIDES DURING THE CLOTTING OF FIBRINOGEN

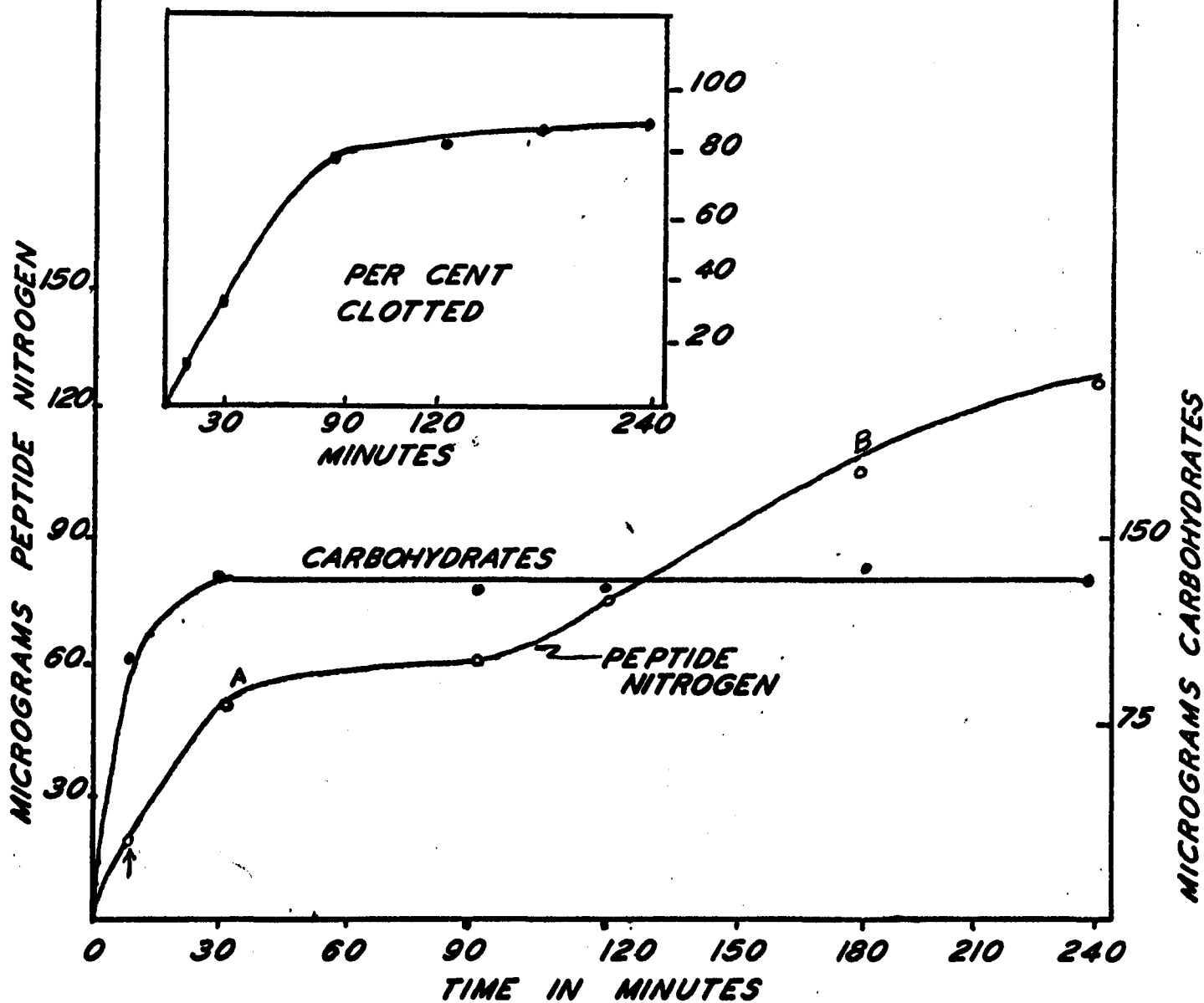


Figure 6. - ASSAY SYSTEM: 16 mgm. fibrinogen/ml. in 0.3 M NaCl:phosphate buffer (1:1), pH 6.4, u = 0.1 and 0.32 NIH Units/ml. of human thrombin. Total volume = 4 ml. Time 0 to 240 minutes at 37°C. ↑ = clotting time (9½ minutes).

stabilizing factor in the fraction I₄ was not reported by Blomback,["] however, in some later experiments it was found that at least 9% of the fibrin which was formed with the fraction I₄ was not soluble in either 1 Molar sodium bromide or 6 Molar urea. A recent communication with Dr. Mosesson has confirmed the author's impression that plasminogen-free fibrinogen preparations would also contain variable amounts of fibrin-stabilizing factor.

f) Effect of ε-amino-caproic acid and sodium heparinate on the activities of thrombin.

As mentioned previously the fibrinogen used in these experiments was said to contain approximately ten per cent of the plasminogen which was present in the starting Cohn fraction I. This plasminogen can be auto-catalytically converted into the active proteinase, plasmin, which will digest fibrin much more readily than fibrinogen. The presence of 10⁻⁴ M ε-amino-caproic acid has been shown to inhibit the conversion of plasminogen to plasmin, however, concentrations of 10⁻² Molar were found necessary to inhibit 90 per cent of the plasmin activity (Alkjaersig, 1959).

The incorporation of 10⁻³ M ε-amino-caproic acid (EAC) into the assay system did not decrease the amounts of peptide nitrogen released at 40 minutes and the total peptide nitrogen released at the end of 240 minutes was only reduced from 178 to 170 ugms., indicating that fibrinopeptide B does not arise from the action of plasmin on fibrin. An exhaustive study which may

implicate plasmin in the release of human fibrinopeptide B was not done. A detailed study of this type requires that plasminogen-free fibrinogen or higher concentrations of EAC be employed in the assay system.

Since ϵ -amino-caproic acid is currently being used in clinical trials to inhibit the action of plasmin in fibrinolytic diseases, it was interesting to find out if the presence of ϵ -amino-caproic acid would inhibit thrombin as well as plasmin and therefore effect the one stage prothrombin time. The Simplastin preparation (Warner-Chilcott Co.) is commonly used for clinical prothrombin determinations and this preparation was made to contain various concentrations of ϵ -amino-caproic acid from 10^{-3} to 10^{-1} . A Simplastin preparation containing 10^{-1} M glycine was taken as an appropriate comparative control. The prothrombin times are given as the average of two separate determinations and the differences of the duplicate determinations was less than 0.3 second. The following Table III illustrates the effects of glycine and ϵ -amino-caproic acid on a normal prothrombin time of 13.0 seconds.

TABLE III

Effects of ϵ -amino caproic acid on the one-stage prothrombin times.

<u>Concentration</u>	<u>Prothrombin Time (seconds)</u>
ϵ - amino caproic acid 10^{-3}	14.4
ϵ - amino caproic acid 10^{-1}	14.4
ϵ - amino caproic acid 10^{-1}	14.4
glycine (control) 10^{-1}	13.5

Eleven citrated plasma specimens were randomly taken from the hospital morning samples and prothrombin determinations were made in the presence of 10^{-1} Molar ϵ -amino caproic acid. The values given in () were obtained in the absence of ϵ -amino caproic acid. The following prothrombin times were obtained: 20.7 (18.0); 13.2 (12.3); 19.0 (18.6); 27.4 (24.2); 25.9 (23.3); 13.6 (12.8); 11.0 (10.6); 21.7 (20.7); 14.4 (13.0); 34.3 (31.0); 19.9 (18.0).

It may be concluded that ϵ -amino caproic acid does not have any marked effects on either the conversion of prothrombin to thrombin by the action of thromboplastin, or the conversion of fibrinogen to fibrin by the action of thrombin.

The previously described assay system, Experimental Section III, part b, was used to study the effects of heparin on the release of peptides from fibrinogen. The stock sodium heparin

solution 10,000 USP Units/ml. (ca. 100 mgm.) Testagar & Co., Lot #12726 was diluted with the pH 6.4, phosphate-NaCl buffer mixture to the concentrations of 100, 300 and 500 USP Units/0.5 ml. The clotting times were prolonged from 9'30" to 12'4", 17'0" and 24'15" respectively when the above concentrations of heparin were present in the assay system. However, none of these concentrations of heparinate added to the above assay system would prevent the eventual formation of a fibrin clot. The fibrin clots could not be completely collected on the glass rods within the stipulated one minute interval allotted for their collection. As much of the clot as possible was removed and the remaining small discrete clots were left to be removed during the precipitation and filtration procedure. None of the above concentrations of heparin showed any pronounced effect on the amounts of peptide nitrogen released after a 40 minute incubation period. The amounts of peptide nitrogen found at the end of the 40 minutes were 55, 58, and 50 ugms. for the above concentrations of heparin as compared with 53 ugms. when heparin was not present. The addition of 300 USP units of sodium heparinate to the assay system at 0, 3 and 6 minutes did not affect the amounts of peptide nitrogen found at the end of the 40 minute incubation period, which was 53, 57, 56 ugms. In later experiments concerning the effects of heparin on fibrin polymerization, it was found that the clots could have been collected and completely recovered by snaring on a stiff nichrome loop.

Therefore heparin retards clot formation in these experiments without affecting the release of either fibrinopeptides A or B.

IV. Some kinetic considerations concerning the polymerization of human fibrin in the presence of heparin

a) Introductory remarks

There are many observations concerning the clotting rates of fibrinogen transformed into fibrin, but the complete kinetic study regarding the thrombolytic conversion of fibrinogen into fibrin monomers and thence into fibrin polymers has not been done. The nature of the polymerization of fibrin has been a subject of lively discussion during the past twenty years, since Laki and Mommaerts (1945) demonstrated that at low pH values that thrombin changed fibrinogen in some way that the coagulation time was shortened after reneutralization. Steiner and Laki (1951) demonstrated by light scattering measurements that fibrin polymerization occurs in two ways, i.e., end-to-end aggregation and lateral aggregation. Ferry, et al (1952) using similar measurements in the presence of 1 M urea at pH 6.3, showed that the fibrin polymer under these conditions consisted of 15 monomers with a thickness of a double monomer, and thue Ferry introduced the term 'side-by-side' aggregation. Waugh and Livingston (1951) were among the first to study the kinetic behavior of what can now be called the "thrombin-fibrinogen-fibrin" system and found that

the formation of fibrin was a pseudo first order reaction.

Blombäck and Laurent (1957) were probably the first to recognize that the fibrinopeptides released from fibrinogen and the polymerization of fibrin were separate kinetic reactions, and had they known that the latent release of the second fibrinopeptide (B) was not a requirement for coagulation to occur as shown by Shainoff (1963); then their molecular weight-light-scattering studies would probably have been done on the conversion of fibrin monomers to polymers. However, they were able to indicate at pH 6.4 and 7.4 that the increase in molecular weight was initially an end-to-end polymerization; whereas, at higher pH values (9.0 and 9.9) the lateral aggregation of fibrin also initially occurred. Therefore, all of the kinetic reaction studies done at pH values near 6, whether the measurements were by light scattering, calorimetric, or UV^{280 mμ} absorption, have indicated that the polymerization of fibrin is apparently a first-order reaction (Sturtevant et al, 1955).

Since the presence of heparin did not appear to retard the release of peptide nitrogen in the experiments (Section III, g), but did delay the appearance of the fibrin clots, it was necessary to make some observations regarding the "monomeric-polymeric" fibrin system. The experimental approach was in many ways similar to the classical experiment of Waugh and

Livingston (1951), who used only the "thrombin-fibrinogen-fibrin" system. In designing the experiment presented in this section, the assumption was made that if equal amounts of monomeric fibrin solutions were made to contain either no heparin or different concentrations of heparin, then any changes in the fibrin polymerization rates would be due to the presence of heparin. Although such variables as actual heparin concentration, volume, ionic strength, and osmotic pressure changes were not made, the data obtained from the measurements of bromide concentrations, fibrin monomers (soluble fibrin), fibrin polymer (fibrin clots) and time were encouraging and are presented as a qualitative index regarding the effects of heparin on the polymerization of fibrin.

- b) The experimental conditions used to measure the polymerization of fibrin monomers in the presence and absence of heparin.

Approximately 2 gms. of fibrin were washed with distilled water and then dissolved in 200 ml. of solution containing 1.0 M NaBr; 0.003 M Na₂HPO₄; 0.012 M KH₂PO₄ and 0.075 M NaCl per liter. The dissolved fibrin was filtered through cotton gauze, and 25 ml. each was placed in three 50 ml. volumetric flasks, #1 containing 1 ml. of the phosphate-NaCl buffer and #2 and #3 containing 1 ml. each of sodium heparinate (50 or 100 mgm.) in the phosphate-NaCl buffer mixture. All of the

flasks were diluted to volume and mixed with the 1.0 M NaBr-phosphate-NaCl mixture. Each fibrin monomeric solution was placed in separate Visking dialysis bags (32/23) which were wired with open glass tube flanges to permit access to the dialysis bag. All three dialysis bags were placed into a liter cylinder containing 950 ml. of the phosphate-NaCl buffer mixture, pH 6.3, so that the contents of each bag was submerged below the liquid level of the cylinder. The contents of the cylinder was continuously mixed and exchanged with the buffer mixture at the rate of 1 liter/65 minutes at 21°C. An aliquot (5 ml.) was removed from each dialysis bag at various timed intervals (0,27,37,47,57, and 67 minutes). If any fibrin clots (fibrin polymers) were formed they were removed prior to the 5 ml. aliquots (fibrin monomers). The fibrin clots and monomeric fibrin solutions were both analyzed for fibrin contents by the alkaline-urea method at UV²⁸⁰ mu. The monomeric fibrin solutions were also analyzed for bromides by the gold chloride method. A graphic representation (Figure 7) indicates that the critical sodium bromide concentration for fibrin polymer formation was 0.41 Molar NaBr. A range of 0.34-0.45 Molar sodium bromide was reported by Shulman (1953) as the minimum inhibiting concentration for the clotting of fibrinogen by thrombin at pH 6.2, ionic strength 0.45. The range suggested by these experiments appears considerably narrower, and is not markedly changed by the presence of sodium heparinate.

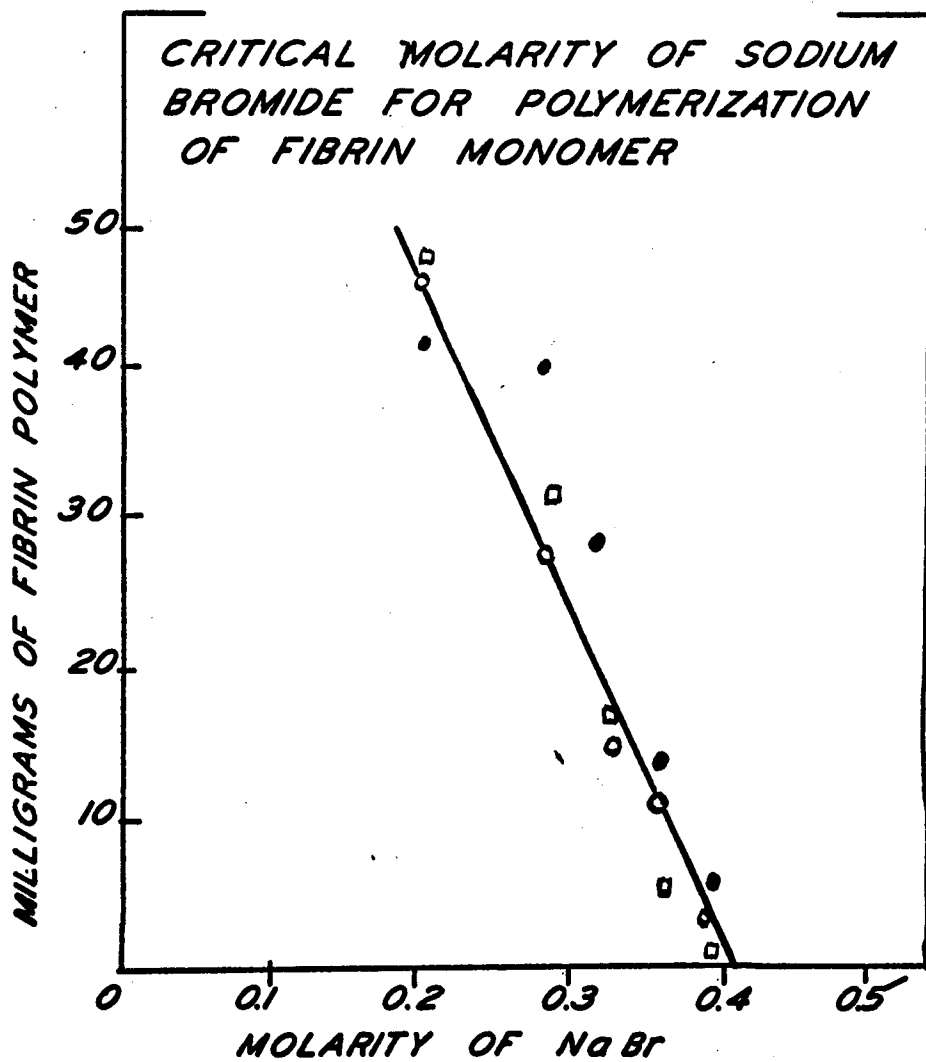


Figure 7.

The amounts of protein present in each clot (expressed as mgm. of fibrin polymer) at the various timed periods are represented in Table IV., along with the parenthetical expressions of the fibrin polymer-monomer ratios. Upon initial inspection of the amounts of fibrin clots formed in the presence of heparin, it would appear that heparin had delayed the formation of the fibrin polymer. However, when the logarithmic function of the fibrin polymer/monomer ratios were plotted against the linear time measurements (Figure 8), it is noted that the polymerization of fibrin proceeds at apparently a first-order kinetic rate, and that the presence of heparinate does not merely depress the initial rate but would appear to alter the kinetics of fibrin polymerization. Since the physico-chemical characteristics of the heparin used in these studies can only be assumed, the absolute evaluation of the kinetic results does not seem permissible.

The kinetic data would tend to support the view expressed by Seegers (1955), which is that heparin interferes with the polymerization reactions. Furthermore, the total amounts of fibrin polymer obtained in samples 2 and 3 at 67 minutes and the accelerated reaction rates at this time would support the view expressed by Shinowara and Everhart (1949), which is that heparin does not inhibit the clotting of pure fibrinogen preparations. In other words, heparin initially depresses the rate of fibrin

TABLE IV

Total amounts of fibrin polymers (clots) obtained during the polymerization of fibrin monomers.

<u>Time (minutes)</u>	<u>Sample #1 (no heparin)</u>	<u>Sample #2 (50 mgm. heparin)</u>	<u>Sample #3 (100 mgm. heparin)</u>
27	5.5 (0.026)	4.4 (0.021)	0.6 (0.003)
37	13.5 (0.078)	10.9 (0.050)	5.8 (0.028)
47	29.3 (0.17)	14.2 (0.07)	16.6 (0.083)
57	39.9 (0.29)	27.1 (0.16)	31.8 (0.17)
67	40.9 (0.41)	45.6 (0.37)	46.6 (0.35)

The milligrams of fibrin polymers (clots) and remaining fibrin monomers were obtained by alkaline-urea UV^{280mu}_{1 cm.} measurements of the samples obtained at various periods of time (minutes) during the polymerization reaction. The parenthetical expressions are the ratio of polymer to monomer at these various times.

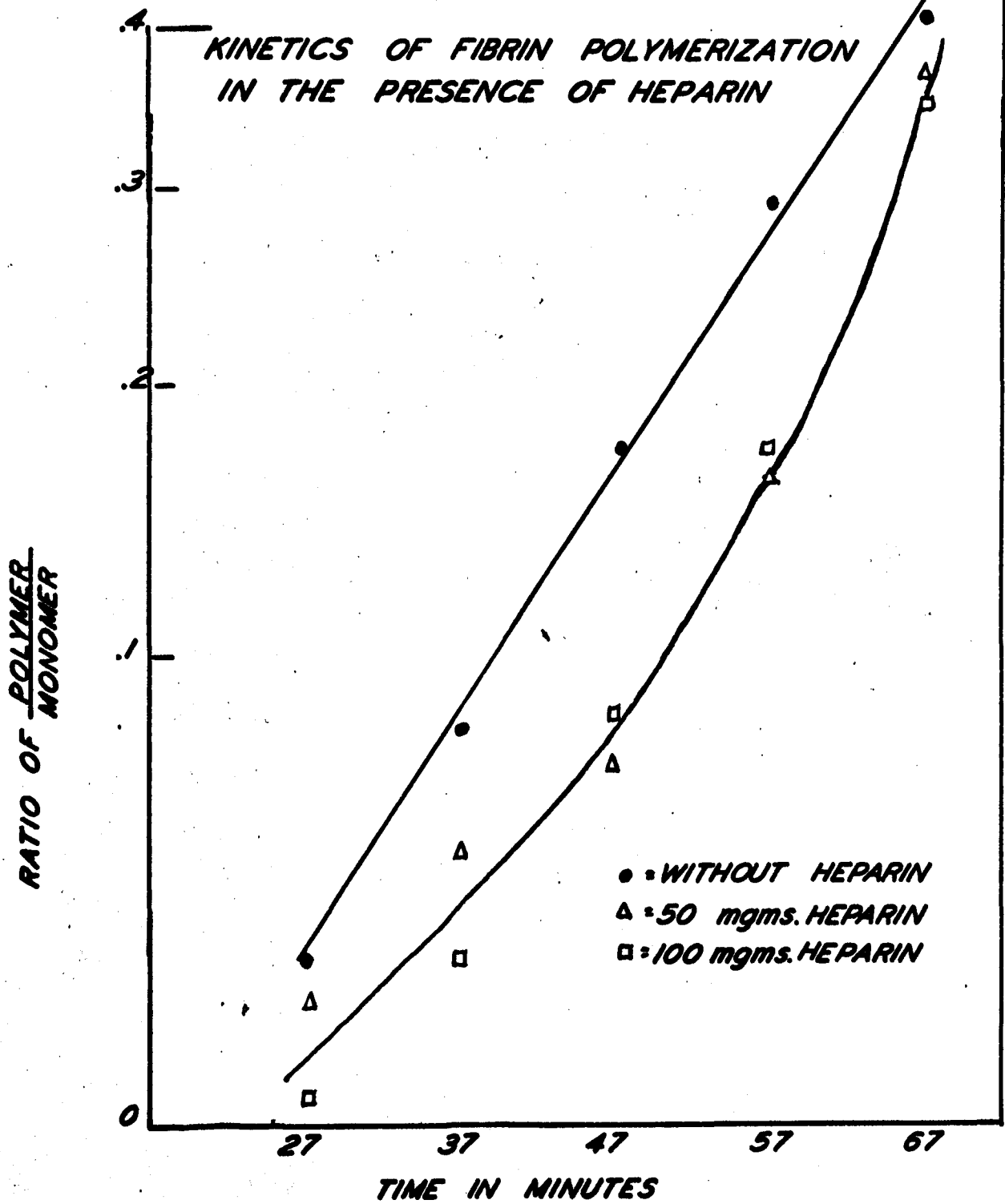


Figure 8. - The initial 50 ml. solutions of 1 M sodium bromide each contained 213 milligrams of fibrin monomer.

polymerization, however the final amounts of polymer formed are the same in the presence or absence of heparin.

V. The effects of heparin on various proteolytic enzymes derived from human plasma

a) Introductory remarks

The previous experiments indicated that the effects of heparin were on the polymerization of fibrin monomers, rather than on the proteolytic activity of the human thrombin preparation used in these experiments. Another experimental approach was undertaken to show that the effects of heparin were not on the enzymatic activity of thrombin. The synthetic substrate chosen was benzoyl-L-arginine ethyl ester (BAEE), which was shown by Sherry and Troll (1954) to be hydrolyzed by both plasmin and thrombin. The synthetic arginyl substrates such as BAEE are not specific for any particular enzyme, however, these substrates are frequently used to indicate the presence of enzymes of the trypsin-like group. Because of the immediate quantitative data obtained with arginyl substrates, they are used to follow the purification steps of trypsin-like enzymes. In the case of plasmin, Ronwin (1961) has shown that the esterase activity of various plasmin preparations on BAEE does not parallel their proteolytic activity, and has suggested the existence of more than one plasmin.

Several years ago the author, Brown (1960), found that

the addition of heparin to normal human sera, oxalated or citrated plasma would increase their arginyl esterase activities.

Forde, et al (1962) utilizing this method were able to report that heparin increased the arginyl esterase activity of oxalated plasma. However, they did not describe any plasma protein fraction as being involved in the "activation effect" of heparin on the hydrolysis of BAEE by human plasma.

The following observations and kinetic data are presented with the realizations, first, that commercial heparin preparations were used, and therefore may not strictly apply to studies conducted with purified human heparin; secondly, that a heparin activated plasma protein fraction which hydrolyzes BAEE and is free of plasminogen, plasmin, and thrombin has not yet been prepared. The author has found that the "activation effect" of heparin can be demonstrated with the commercial thrombin-enriched, plasmin-poor fraction of plasma proteins which represents less than two per cent of the total plasma proteins.

b) The relationship of heparin concentrations to the hydrolysis of BAEE and acetylcholine by plasma and sera

The details of these experiments were as follows:

The pooled oxalated human plasma and serum samples were obtained from five hospital blood donors. The word "pooled" designates that these samples were used in the experiments. Other experiments were conducted on randomly collected serum

samples and received no special designation.

The alkaline hydroxamate-ferric chloride colorimetric method of Brown (1960) was used to determine the μ Moles of BAEE or acetylcholine hydrolyzed per milliliter of plasma or serum per hour. The assay system was made to contain between 4 and 100 μ gms. of sodium heparinate (Testagar & C., Lot #12726) per ml. in a total volume of 2.5 ml. The relationship of heparin concentrations to BAEE hydrolysis by the pooled plasma and sera are shown in Figure VI. Heparin concentrations greater than 40 μ gms. and up to 100 μ gms. per ml. resulted in approximately a two-fold increase of the sera or plasma arginyl esterase activities. The activation effect of heparin on the sera arginyl esterase activity appears to be slightly greater than on the corresponding plasma sample. Incubated substrate controls containing similar heparin concentrations without plasma or sera did not demonstrate any arginyl esterase activity. Furthermore, four heparin preparations obtained from different manufacturers were all found to increase the serum arginyl esterase activity in an equivalent manner. These heparin preparations contained either chlorobutanol, phenol, or benzyl alcohol as an added preservative. The addition of 100 μ gms. of phenol instead of heparin did not result in an increase of the serum arginyl esterase activity. These results were taken to mean that the "activation effects" of heparin were not restricted to any one

particular heparin preparation, and were not due to the preservative added.

The acetylcholine hydrolysis by serum was 162 μ Moles per ml. per hour and was not significantly changed by heparin concentrations up to 200 μ gms. per ml. These results would suggest that the effects of heparin were limited in its serum esterase activation. The BaSO_4 or $\text{Ca}_3(\text{PO}_4)_2$ treatment of plasma or serum is said to remove both thrombin and prothrombin. Several serum samples (9) were separately mixed with 150 mgm. of BaSO_4 per ml. of serum and allowed to stand for 10 minutes. The BaSO_4 was removed by centrifugation. There was found a variable decrease of the BaSO_4 -treated serum arginyl esterase activities which averaged 6 μ Moles per ml. per hour.

The pooled sera were treated with 200 mgm. of $\text{Ca}_3(\text{PO}_4)_2$ per ml. of sample and allowed to stand for 10 minutes before centrifugal removal of the $\text{Ca}_3(\text{PO}_4)_2$. The $\text{Ca}_3(\text{PO}_4)_2$ -treated-sera was found to decrease the arginyl esterase activity from 25.5 to 14.8 μ Moles per ml. per hour. The effects of heparin (10, 20, 40, 60 and 100 μ gms.) on the arginyl esterase activity of the $\text{Ca}_3(\text{PO}_4)_2$ -treated-sera were studied and the results are shown in Figure VI. The optimum concentration of heparin for the maximal increase of arginyl esterase activity was found to change from 40 μ gms. per ml. to approximately

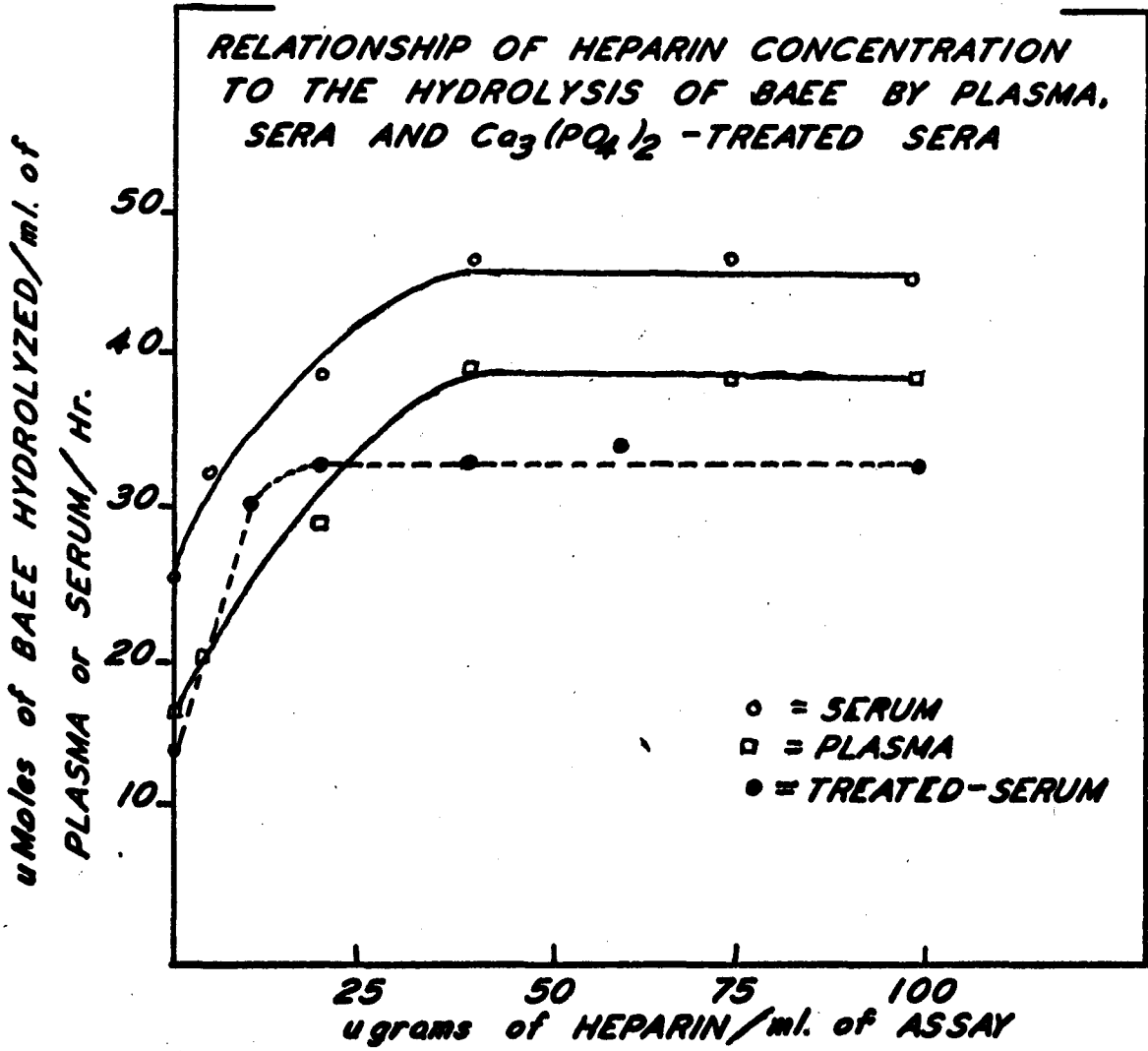


Figure 9.

10 ugms. per ml. following $\text{Ca}_3(\text{PO}_4)_2$ treatment, however, the two-fold increase of activity due to the addition of heparin remained. These results were suggestive that human thrombin was not involved in the heparin "activation effect" on the serum arginyl esterase activity.

c) The effects of heparin on the enzymatic activities of crude and column purified human thrombin

Various amounts of crude human thrombin or column (Cellex-P) purified human thrombin (courtesy of Dr. J. Inman, Ortho Research Laboratories) were assayed for arginyl esterase activities. The purified human thrombin was said to be free of plasminogen and plasmin. The addition of 1,000 to 5,000 Units of streptokinase (Varidase) failed to increase the arginyl esterase activities of either the crude or purified human thrombin preparations. As shown in Figure VII, the uMoles of BAEE hydrolyzed per hour were related to the NIH clotting Units in both preparations. The purified thrombin preparation was slightly more active than the crude thrombin, in terms of their arginyl ester hydrolysis per NIH Units of thrombin.

The relationship of various heparin concentrations to the esterase activities of these two thrombin preparations were remarkably different as shown in Figure VIII. Various amounts of heparin did not demonstrate an "activation effect" on the purified thrombin enzymatic activity, whereas, the effect of

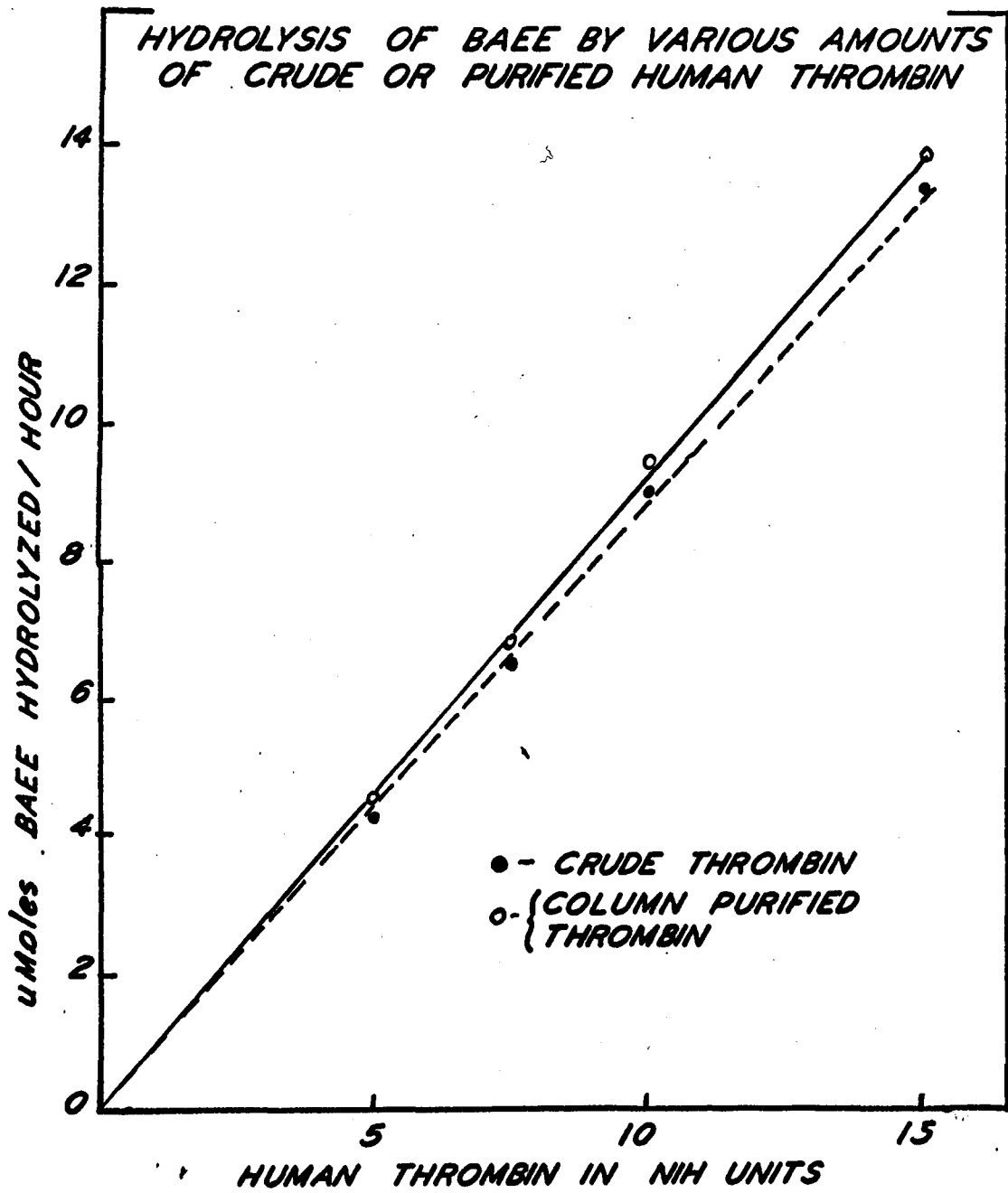


Figure 10.

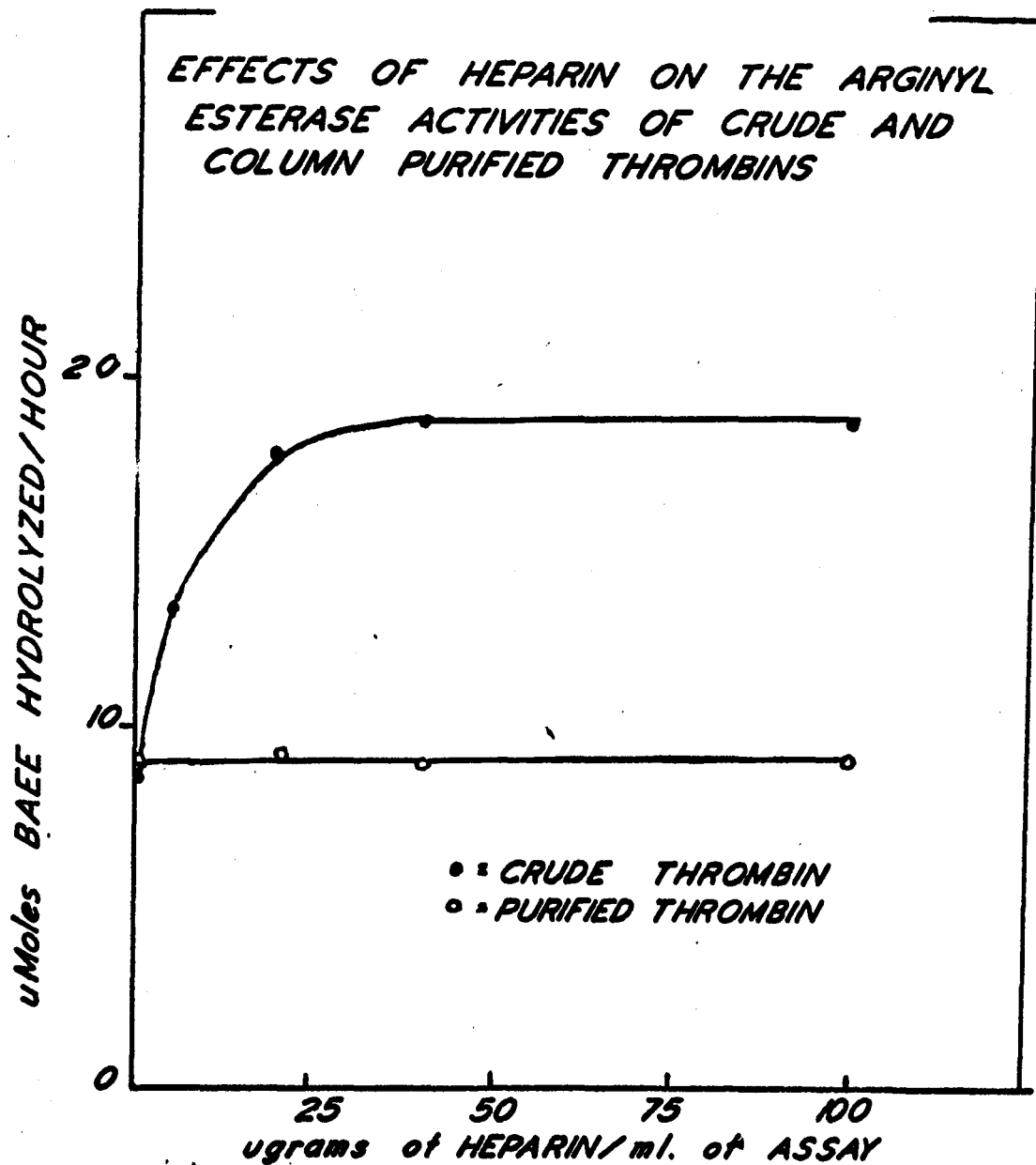


Figure 11. - Ten NIH Units each of either crude or Cellex-P column purified human thrombin was used in each assay.

heparin on the enzymatic activity of the crude thrombin preparation was similar to that observed with human plasma or serum.

These results in conjunction with those obtained with plasma and serum would support a suggestion that a heparin dependent enzyme (capable of hydrolyzing BAEE) is present in the plasma, serum, and crude thrombin preparations, and that this enzyme is not thrombin, per se.

Similar observations regarding the "activation effects" of heparin on commercial bovine thrombin preparations (Upjohn Co. and Parke-Davis Co.) have also been made by the author. Blomback and Blomback (1958) have shown that the esterase activity of purified bovine thrombin, as measured on tosylarginine methyl ester, was not inhibited by heparin alone, however, they did not indicate the effects of heparin on the crude bovine thrombin preparation.

The author has been unable to demonstrate the heparin "activation effect" with either commercial plasminogen or plasmin preparations, and has not been able to activate plasminogen with purified thrombin in either the presence or absence of heparin. A competitive inhibition between plasmin and purified thrombin for the BAEE substrate can be demonstrated, and a similar type of inhibition has been reported by Pechet and Alexander (1962) using fibrinogen as the substrate. Heparin additions had no effect on the competitive inhibition between plasmin and thrombin for the substrate BAEE.

DISCUSSION AND SUMMARY

The criterion of the clottability of fibrinogen is an additional parameter of protein purity which is not shared by other plasma proteins. This unique clotting characteristic of the fibrinogen molecule can be readily altered by enzymatic and chemical treatment. Mihalyi (1963) has shown that the controlled tryptic cleavage of a single peptide bond will render the molecule non-clottable. The acetylation of 35 per cent of the free amino groups (Caspary, 1956), or the guanidination of 75 per cent of the lysyl groups (Kominz and Laki, 1954), or the iodination of the tyrosyl groups (Laki and Steiner, 1952) of fibrinogen were found to produce a non-clottable molecule. Laki and Mester (1962) reported that the treatment of fibrinogen with periodic acid for various periods of time would result in a gradual decrease in the clottability, and the sialic and carbohydrate contents of the molecule. The experiments presented in this dissertation indicated that the controlled hydrazine treatment of fibrinogen would result in the formation of a fibrinogen-hydrazide which was non-clottable. The ultracentrifuge comparative study of the treated and untreated fibrinogen suggested that the major portion of the molecule remained intact. The spectrometric evidence has indicated that the fibrinogen-hydrazide contains three moles of heat-labile hydrazine per mole of fibrinogen. The definitive reports of the Gallop group concerning the conditions for the hydrazine reaction with ester-like linkages

were followed in these experiments on the human plasma proteins and fibrinogen was found to be the only plasma protein to demonstrate any extensive reaction with hydrazine. This would suggest that ester-like linkages are present in fibrinogen. Bergmann, et al. (1939) suggested but were unable to exclude the possibility of ester or ester-type linkages in the fibrinogen molecule. The failure of α_1 -glycoprotein to react with more than 0.32 moles of hydrazine per mole of protein would exclude the possibility that the hydroxyl groups of the carbohydrates had reacted with hydrazine under the conditions used in these experiments. And it would tentatively exclude the possibility that the carbohydrate peptide linkage of α_1 -glycoprotein was of an ester-type. Due to the larger amounts of hexoses in α_1 -glycoprotein (16%) as compared with fibrinogen (1.6%), it would seem reasonable to suppose that the carbohydrate-peptide bonds of the former protein were not readily accessible to react with hydrazine, whereas the carbohydrate peptide bonds of the latter protein were accessible. It will be of some interest to test this possibility either, by a longer treatment of α_1 -glycoprotein, or

the controlled hydrazine treatment of ovalbumin (1.8% hexoses). The finding of only 0.2 moles of heat-labile hydrazine per mole of hydrazine-treated human albumin would suggest that the ester-like linkages are not found in the molecular structure of this protein. The lack of ester-like linkages in albumin would suggest that those found in fibrinogen were not due to its secondary or tertiary structure. The probable existence of one ester-bond per mole of bovine albumin has been suggested by Gallop (1962a), but no further reports have appeared to validate this communication.

The carbohydrates of fibrinogen are not generally recognized, however, the chromatographic demonstration of mannose, galactose, hexosamine, and sialic acid in these experiments has firmly established fibrinogen as a glycoprotein. The percentages of hexoses, hexosamines, and sialic acids found in the fibrinogen preparation used in these experiments differ from those reported by Blomback["] for bovine fibrinogen (see page 15). The hexose and hexosamine values are in agreement with those reported by Bagdy and those currently being obtained by the NIH group (Laki and Mester, 1962). The sialic acid content disagrees with the value of "none" reported by Bagdy and somewhat with the value of 0.6% currently being found by the NIH group. The difference in the amounts of sialic acids is probably due to the difference in the methods used to determine sialic acids. Based on the values of the various

carbohydrate components it was calculated that human fibrinogen (M.W.=340,000) would contain approximately 29 residues of neutral hexoses (M.W. galactose=180); 9 residues of N-acetyl hexosamines (M.W.=221); and 10 residues of sialic acid as N-acetyl neuraminic acid (M.W.=309). During the molecular conversion of fibrinogen to fibrin there would be released approximately 5 residues of hexoses, probably as galactose moieties and 1 residue of N-acetyl neuraminic acid.

The finding of differences in the dialyzable reducing substances of the paired serum and plasma analyses and the indication that hexoses are released during the conversion of fibrinogen to fibrin would support the hypothesis that hexose-release was involved in the blood coagulation mechanism. Some of the differences between the paired serum-plasma analyses are probably due to the conversion of prothrombin to thrombin. Miller and Seegers (1956) reported that during the conversion of prothrombin to thrombin, there was released 40 to 60 per cent of the hexoses of prothrombin (presumably as tetrasaccharides consisting of glucose moieties).

The release of peptides during the conversion of fibrinogen to fibrin has already been established. However, the clear indication that human fibrinopeptide B release occurs after the formation of fibrin has only recently been reported (Brown, 1962).

The release of fibrinopeptide B did not appear to involve proteolytic activities of plasmin in these experiments. The recent reports of Lorand, et al (1962) indicated that the role of the fibrinestablizing-factor (FSF) was one of transpeptidation; it would be interesting to speculate that fibrinopeptide B release was associated with FSF. Some preliminary experiments concerning the electrophoretic comparisons of human and bovine fibrinopeptides indicated that both of the human fibrinopeptides A and B were different from those serived from bovine fibrinogen. A review of the reports by Blomback["] and Sjoquist["] (1960), Shainoff and Page (1960) and Doolittle et al (1962) clearly indicated the marked electrophoretic differences of bovine, rabbit, lamprey eel, sheep, and dog fibrinopeptides. Our Sakaguchi-reaction for arginine has not been stablized enough to permit photographic reproductions. However, under the paper electrophoretic conditions which were similar to those used by the above authors, we found that both of the human fibrinopeptides migrated toward the positive pole. The mobilities of the human fibrinopeptides A were similar to those reported for eel fibrinopeptides A, and that the migration of human fibrinopeptides B were similar to those reported for dog fibrinopeptides B. The human fibrinopeptides B yielded about a two-fold stronger Sakaguchi positive area than the positive area of fibrinopeptides A.

The role of heparin on the fibrinogen, fibrin monomer, and thrombins used in these experiments suggests that: (a) heparin does not affect the proteolytic or esterase activities of purified thrombin; (b) fibrin monomers are probably formed in the presence of heparin; (c) at the milligram ratio of 1:2 (heparin:fibrin monomer) an interaction occurs between heparin and fibrin monomer which delays the polymerization of the fibrin monomers; (d) microgram quantities of heparin have an "activation" effect on the arginyl esterase of crude but not purified human thrombin preparations. It is tempting to associate the two types of aggregation of fibrin monomers with the possible effects of heparin, that is, the end-to-end association occurring primarily in the absence of heparin and the secondary side-by-side association occurring in the presence of heparin.

In conclusion, a number of the planned studies concerning the release of carbohydrates and peptides during the conversion of fibrinogen to fibrin were reduced, because of the limited supply of column purified thrombin which was available. The necessity of using proteins which are highly purified is obvious, especially concerning studies of such a complex biochemical mechanism as blood coagulation.

SUMMARY:

1) Both human and bovine fibrinogens react with buffered hydrazine to form a non-clottable fibrinogen-hydrazide. The spectro-metric analyses indicated that three moles of "heat-labile" hydrazine per mole of human fibrinogen were formed during the reaction. Human albumin and α_1 -glycoprotein were also found to react with buffered hydrazine to form less than 0.5 mole of "heat-labile" hydrazine per mole of protein. The results suggest that there are ester-like bonds in the primary structure of fibrinogen.

2) The "Ehrlich's reaction" used with the above proteins and others (insulin, lysozyme, trypsin, chymotrypsin, and bovine albumin) indicated a spectral characteristic absorption curve at wavelengths between 630 and 580 millimicrons which are associated with the number of tryptophyl residues per mole of the respective protein.

3) The carbohydrate components of human fibrinogen (fraction I₄) prepared according to Blomback and Blomback (1956) were determined as: 1.6% hexoses; 0.6% hexosamines; and 1.0% sialic acids. The calculated carbohydrate residues per mole of fibrinogen (M.W. 340,000) are approximately: 29 residues of hexoses; 9 residues of hexosamines; and 10 residues of sialic acids. The fibrin monomer contains approximately 5 and 1 residue(s) less of hexoses and sialic acid, respectively. The hexoses were identified from fibrinogen and fibrin digests as mannose and galactose (approximately equal amounts of each). The type of sialic acid in fibrinogen was similar to that

obtained from the human α_1 -glycoprotein preparation.

4) An assay system was described which indicated that the release of carbohydrates and peptide nitrogen occurred during the conversion of human fibrinogen to fibrin. The latent release of the human peptide nitrogen component B suggested that fibrinopeptide B was not associated with the formation of fibrin polymer (clot). The rate of peptide nitrogen A release was associated with the formation of the fibrin polymer (clot). The peptide nitrogen A release was not altered by various amounts of heparin added to the assay system.

5) The addition of heparin to fibrin monomers dissolved in 1 Molar sodium bromide and then dialyzing-out the sodium bromide under controlled conditions permitted a study of fibrin polymerization. The studies indicated that the presence of heparin had a profound effect on the apparent first-order kinetics of fibrin polymerization which were observed in the absence of heparin. A critical sodium bromide concentration for fibrin polymerization was found at 0.41 Molar sodium bromide.

6) The addition of heparin in microgram amounts to the arginyl esterase measurements of purified thrombin activities had no effects on the esterase activity of this thrombin preparation. However, an "activation effect" of heparin on the arginyl esterase activities of serum, plasma, calcium phosphate-treated serum and crude thrombin preparations was observed.

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ABSTRACT

THE RELATIONSHIP OF CARBOHYDRATES AND PEPTIDES IN HUMAN FIBRINOGEN AND FIBRIN

Three purified human plasma proteins, albumin, α_1 -glycoprotein (prepared according to Schmid) and 96 per cent clottable fibrinogen (prepared according to Blomback, fraction I₄) were found to react with 1 M hydrazine at pH 9.0 under conditions similar to those used by Gallop, et al. for determining the ester-like bonds in collagen. The moles of "heat-labile" hydrazine per mole of each human plasma protein studied were approximately: 0.2 for albumin, 0.3 for α_1 -glycoprotein, and 3.4 for fibrinogen. The spectral absorption curves of the "heat-labile" hydrazine derived from the treated proteins were comparable with those obtained for the hydrazine standards. The kinetics of reaction time and the pH optimum for the formation of fibrinogen-hydrazide were studied. The small amounts of hydrazine reacted with α_1 -glycoprotein would suggest that the hydroxyl groups of the carbohydrate moiety of glycoproteins are not involved in the hydrazine reaction used in these experiments. The amounts of hydrazine reacted with albumin would suggest that the hydrazine reaction with the plasma proteins used in this investigation is unique for fibrinogen.

Some preliminary physico-chemical characteristics of the fibrinogen-hydrazide preparations were: a) the complete loss of clottability as compared with only 12 per cent loss in the clottability of the control treated fibrinogen preparation, b) paper

electrophoretic mobility of fibrinogen-hydrazide was similar to that of the original fibrinogen preparation, c) a single comparative ultracentrifuge run indicated the homogeneity of the original fibrinogen preparation suggesting a peculiar aggregation of the fibrinogen-hydrazine preparation which apparently sediments similar to the original fibrinogen preparation.

The "Ehrlich's reaction" used in these experiments was applied to several carbohydrates, amino acids, and other proteins (insulin, lysozyme, chymotrypsin, trypsin, and bovine albumin), and resulted in characteristic spectral absorption at wavelengths between 630 and 580 millimicrons related to the number of tryptophanyl residues per mole of the respective protein.

The analyses of carbohydrate components of human fibrinogen preparations indicated approximately: 29 residues of hexoses (chromatographically identified as approximately equal amounts of mannose and galactose); 9 residues of hexosamines; and 10 residues of sialic acids (chromatographically similar to those obtained from α_1 -glycoprotein). Fibrin contains approximately 5 to 6 and 1 to 2 residue(s) less of hexoses and sialic acids, respectively. It is suggested that human fibrinogen be considered as a plasma glycoprotein.

An assay system is described which indicates that carbohydrates and peptide nitrogen are released during the conversion of human fibrinogen to fibrin. The latent release of peptide nitrogen B suggests that fibrinopeptide B is not associated with the formation

of fibrin polymer (clot). However, the rate of peptide nitrogen A could be related to the per cent of clot formed. The carbohydrate release appears to be concomitant with the release of peptide nitrogen A.

The incorporation of 10^{-3} M of ϵ -amino caproic acid (an inhibitor of plasmin activity) into the assay system did not significantly affect the release of either peptide nitrogen A or B. The negative effects of ϵ -amino caproic on clinical prothrombin times were consistent with these results. The addition of 50 to 100 milligrams of heparin (sodium salt) at various times into the assay system delayed the clotting times but did not effect the release of peptide nitrogen A.

The addition of 50 or 100 milligrams of heparin to fibrin monomers (213 mgm.) dissolved in 1 M sodium bromide permitted the preliminary study of fibrin polymerization, and the critical sodium bromide concentration for fibrin polymerization was found at 0.41 Molar sodium bromide. The studies indicated that the presence of heparin had a profound retarding effect on the apparent first-order kinetics of fibrin polymerization which was observed in the absence of heparin.

The addition of heparin in microgram amounts to the arginyl esterase measurements of column purified thrombin activities had no effect on the esterase activity of this thrombin preparation. However an "activation effect" of heparin resulting in an increase of the arginyl esterase activities of serum, plasma, calcium phosphate-treated serum and crude thrombin preparations were observed.

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