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"INVESTIGATION OF THE MECHANISM OF FIBRINOLYSIS"

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Investigations of the mechanism of fibrinolysis through evaluations of the role of enzymes and enzyme inhibitors. Specifically studies were made utilizing the enzyme ribonuclease to inhibit the lysis of fibrin clots (bovine and human) by bovine and/or human plasmin (fibrinolysis).

It has been found that the optimum pH range for effective inhibition of plasmin by ribonuclease is from 8-9. Increases in temperature were found to increase plasmin activity but tend to reduce the inhibitory action of ribonuclease on plasmin activity.

Data obtained indicates that lysis of a fibrin clot by plasmin occurs in two steps: (a) depolymerization of the fibrin clot and (b) subsequent hydrolysis of the depolymerized fibrin to amino acids. Both steps are inhibited by ribonuclease via a plasmin - ribonuclease complex not involving the moiety of the ribonuclease molecule involved in ribonucleic acid degredation. Moreover it was found that dilute acid hydrolysis of bovine pancreatic ribonuclease results in a loss of the nuclease activity of ribonuclease but not its anti-plasmin activity.

Studies made on the role of heparin and ribonuclease in fibrinolysis indicate that heparin inhibits the antiplasmin activity of ribonuclease. In addition it has been observed that bovine pancreatic ribonuclease will inhibit the liquifaction of fibrin clots *in vitro* by heparin in low concentration (1-10 NIH units) in the absence of plasmin.

No other components of the fibrinolytic system studied were observed to possess ribonuclease activity.

The data obtained are discussed in terms of the mechanism of fibrinolysis and the roles and functions of ribonuclease and heparin.

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INTRODUCTION

Based on observations made in this laboratory that bovine pancreatic ribonuclease will inhibit *in vitro* the dissolution of bovine and human fibrin clots by plasmin of either human or bovine origin investigations directed toward elucidation of the mechanism by which plasmin (fibrinolysin) hydrolyzes fibrin clots were made.

The initial stages of this program required modification of existing assay techniques for assessing fibrinolytic activity. As a result a tube test-method was developed and adopted as the method of assaying for fibrinolytic activity to replace the time consuming and expensive fibrin plate method of Astrup and Mullertz (1). This report describes this method and its subsequent usage in studying the effects of temperature, pH, varying concentrations of enzymes, substrates, and inhibitors, etc. on the fibrinolytic process in the presence and absence of ribonuclease and other known inhibitors of fibrinolysis.

In addition and in order to gain insight in general into the mechanism by which fibrinolysis occurs and specifically into the role of ribonuclease in the fibrinolytic mechanism a special four-way program of investigation was undertaken. One series of studies was designed to ascertain whether any of the components of the fibrinolytic system utilized in our work possessed ribonuclease activity, per se, as manifested through depolymerization of ribonucleic acid.

Simultaneously investigations were made to elucidate the means by which bovine pancreatic ribonuclease inhibited the depolymerization and subsequent hydrolysis of bovine fibrin clots by both human and bovine plasmins. Concurrently and in order to assist in pin-pointing the exact function of ribonuclease in the fibrinolytic system experiments were performed in which the role of heparin, reported inhibitor of ribonuclease enzyme activity, was ascertained with respect to its relationship to ribonuclease and the fibrinolytic system.

The fourth part of the general research plan designed to evolve knowledge as to the mechanism of fibrinolysis revolved around the question posed regarding the requirement of the ribonuclease molecule *in toto* for antiplasmin activity.

The methodology used, results obtained, and conclusion to be derived from this research program with respect to evolving the mechanism of fibrinolysis and the specific function of ribonuclease from the period from November 1961 through April 1964 are presented in the following pages of this report.

TEST TUBE ASSAY METHOD

A method was developed for studying the fibrinolytic system which could be carried out in test tubes and with extreme accuracy, precision, and reproducibility. Moreover the assay could be conducted in total volume of 1.0 ml and utilized only 5 mgs of fibrinogen and microgram quantities of enzyme and/or inhibitors. This method as described below is being used now in all studies and was used in the investigations as reported herein.

I - Description of test tube method

To a 10 x 75 mm test tube is added 0.6 ml of 0.0036 mm veronal buffer at desired pH (0.6570 gms diethylbarbituric acid/liter of .9% saline).

Next is added 0.1 cc of fibrinogen solution (5 mg of fibrinogen, bovine or human, per 0.1 ml of veronal buffer). This is followed by 0.1 ml of inhibitor dissolved in veronal buffer or 0.1 ml buffer alone if no inhibitor is being used. Next 0.1 ml of veronal buffer containing the desired concentration of plasmin is added. Finally 0.1 ml of thrombin dissolved in veronal buffer (10 NIH units/0.1 ml) is added. The contents of the tube are mixed after the addition of each solution. Immediately after addition of the thrombin the tubes are mixed and allowed to stand for 1-2 minutes until a fibrin clot forms. The assay tube is then incubated under any desired condition of temperature in a water bath until the fibrin clot has become completely liquified (lysed). The time of complete liquification (lysis) is recorded as the lysis time. If lysis does not occur in 24 hours the enzyme under investigation is considered to be non-fibrinolytic in activity.

Utilizing this method inhibitory action of bovine ribonuclease in an all bovine fibrinolytic system was assessed. All these studies were made at pH 7.8 and 37.0°C. The results of these assays have been summarized in Table I and II. Table I presents data obtained with varying amounts of plasmin and no added ribonuclease. Table II shows the average results of replicate assays in which the concentration of ribonuclease was varied and the concentration of plasmin kept constant at 100 micrograms.

TABLE I

Lysis time of Bovine Plasmin
at
pH 7.8 and 37.0° C

Concentration of plasmin in micrograms	Lysis time in minutes
50	13.0
100	8.0
200	6.0
500	5.0
1000	3.5

TABLE II

Lysis Time of Bovine Plasmin + Bovine Ribonuclease
at
pH 7.8 and 37.5° C

Concentration in micrograms		Lysis time in minutes
Plasmin	Ribonuclease	
100	0	8.0
100	50	9.5
100	100	14.0
100	200	120.0
100	500	No Lysis
100	1000	No Lysis

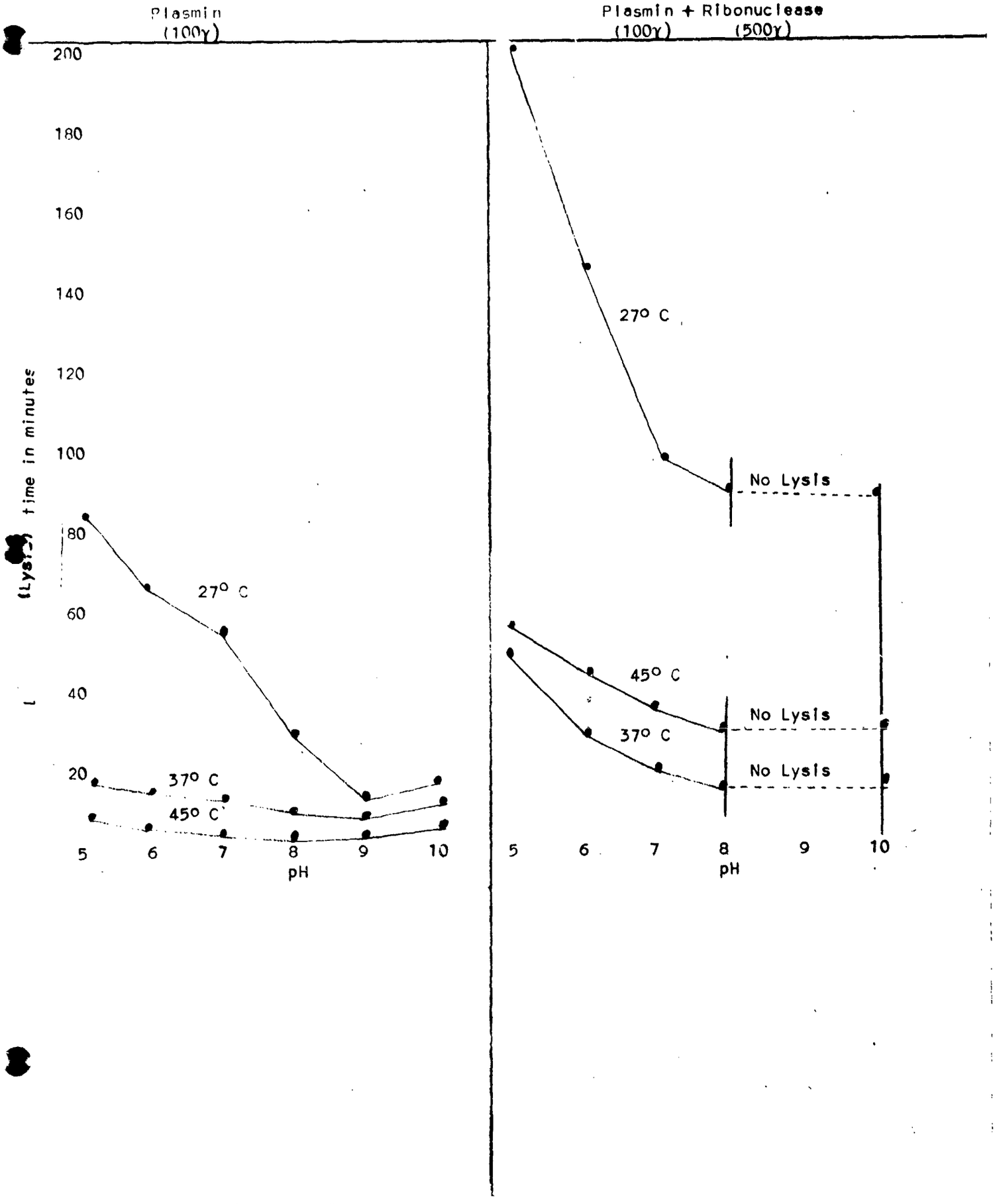
2 - Studies on inhibition of plasmin by ribonuclease

(a) Temperature and pH effects.
A series of experiments were conducted to determine the effect of temperature and pH on the inhibition of plasmin activity by ribonuclease. In these experiments the plasmin was use at a constant concentration of 200 micrograms and the ribonuclease concentration used was kept constant at 1000 micrograms thus maintaining the minimum inhibitory ratio of ribonuclease to plasmin at 5:1. The test tube assay method previously described was used and in all bovine fibrinolytic system was studied. Temperature and pH were varied. The results of these experiments are graphically depicted in Figure I.

(b) Mode of action of ribonuclease inhibition of plasmin
In another series of studies experiments were carried out utilizing the test tube assay method to determine the mode of ribonuclease inhibition of plasmin. These investigations were made to ascertain which component of the fibrinolytic system was affected by ribonuclease directly and resulting in the inhibition of the lysis of a fibrin clot by plasmin. In the first series of experiments varying amounts of bovine ribonu-

FIGURE 1

EFFECT OF TEMPERATURE AND pH ON LYSIS



clease dissolved in 0.1 mo of 0.0036 m veronal buffer pH 7.8 was added to 0.1 ml of veronal buffer (0.0036 m - pH 7.8) containing 5 mg bovine fibrinogen in 10 x75 mm test tubes. The tubes containing the mixtures of ribonuclease and fibrinogen were then incubated at 37.0° C for 30 minutes in a water bath.

Following incubation 0.1 ml veronal buffer containing 50γ of bovine plasmin, 0.6 ml veronal buffer, and 0.1 ml thrombin (10 NIH units) were added to each tube and a fibrin clot allowed to form. The tubes were then incubated at 37.0° C and the time of lysis recorded.

In a second series of experiments 50γ amounts of plasmin were incubated for 30 minutes at 37° C with varying amounts of ribonuclease prior to the formation of the fibrin clot and subsequent incubation of the clot system at 37° C.

In the third set of experiment 50γ amounts of plasmin and 5 mg amounts of fibrinogen were incubated together for 30 minutes prior to fibrin clot with thrombin, and incubation of the clot system at 37° C. The results of these studies are summarized in Table III.

TABLE III

	Lysis Time in Minutes		
	(1)	(2)	(3)
Ribonuclease In micrograms	Ribonuclease + Fibrinogen (5mg)	Ribonuclease + Plasmin (50γ)	Plasmin (50γ) + Fibrinogen (5mg)
0	10	5	5
50	10	50	5
150	10	65	5
500	75	No Lysis	75
1500	No Lysis	No Lysis	No Lysis
3000	No Lysis	No Lysis	No Lysis

Key:

(1) Preincubation of Ribonuclease and Fibrinogen at 37° C for 30 minutes.
(2) Preincubation of Ribonuclease and plasmin (50γ) at 37° C for 30 minutes.
(3) Preincubation of plasmin (50γ) and fibrinogen (5mg) at 37° C for 30 minutes.

(c) Comparison of inhibitors of plasmin.
In another series of experiments utilizing the test tube dilution method the ability of epsilon amino caproic acid and deoxyribonuclease to inhibit bovine plasmin activity were evaluated. All experiments were made at 37.5° C and at pH 7.8. In these experiments a constant amount of bovine plasmin (200γ) was used and the concentration of the inhibitors varied. The results

of these experiments have been summarized in Table IV.

TABLE IV

Inhibition of Plasmin by E-amino Caproic Acid and Deoxyribonuclease at pH 7.8 and 37° C

Concentration of Inhibitor in micrograms	Lysis time in minutes	
	Deoxyribonuclease	E-amino Caproic Acid
0	5	5
50	5	5
100	5	15
200	5	30
500	5	45
1000	5	80
5000	5	No Lysis

INVESTIGATION OF THE RIBONUCLEASE ACTIVITY OF THE FIBRINOLYTIC SYSTEM

The various component biological materials comprising the fibrinolytic system used in our work were evaluated for ribonuclease activity. Evaluations were carried out according to the method of Anfinsen, et. al. (2) as described by Glick (3). Ribonuclease determinations were made at pH 5, 7.8 and 8.6. Systems studied were bovine plasmin, bovine ribonuclease, bovine plasmin + bovine ribonuclease, bovine trypsin, pancreatin (hog), human plasmin, and human plasmin + bovine ribonuclease.

The procedures employed in this phase of the research effort were as follow:

> To 1.0 ml of substrate solution dissolved in appropriate buffer (0.1 m acetate buffer, pH 5.0 or 0.1 m veronal buffer, pH 7.8 and pH 8.6) is added 1.5 ml of appropriate buffer solution containing the enzymes to be evaluated in concentrations from 0-14 mg. The substrate utilized is an 0.8% solution of purified yeast ribonucleic acid in the desired buffer. The solution of enzyme and substrate is allowed to incubate for 25 minutes at 25°C. All solutions are equilibrated at 25°C prior to use. At the end of the incubation period 0.5 ml of precipitating reagent (0.75% uranyl acetate in 25% perchloric acid) is added to the solution of enzyme and substrate to stop the reaction. The precipitate is removed by centrifugation. Then 0.1 ml of the supernatent fluid is added to 3.0 ml of doubly distilled water and the solution read at 260 mμ in the spectrophotometer. Correction is made for the reagent blank measured by the same procedure but without enzyme.

Since the purpose of this phase of the study was to determine if the other components in the fibrinolytic system under investigation possess ribonuclease activity to any degree and if plasmin inhibits ribonuclease activity, no attempt was made to assess the data in terms of units of ribonuclease activity. As a result the data given in the table below, the results of triplicate assays, show ribonuclease activity as transmittance. Since the nature of this assay measures the product of ultra violet absorbing low molecular nucleotide derivatives from digestion of ribonucleic acid by ribonuclease less transmittance of ultra violet light will occur with increased digestion of ribonucleic acid. The results of this work are shown in Table V.

TABLE V

Measurement of Ribonuclease Activity

Enzyme System	Enzyme Concentration (Micrograms)	% Transmission (260 Mμ) pH 5.0	pH 7.8	pH 8.6
A. Bovine Plasmin + Bovine Fibrin				
Ribonuclease[1]	5	47.0	33.0	52.0
Plasmin[2]	5	100.0	90.0	100.0
Plasmin + Ribonuclease (10 : 1)	50:5	43.0	28.2	40.0
Plasmin + Ribonuclease (1 : 1)	5:5	50.0	24.0	56.0
Plasmin + Ribonuclease (1 : 10)	0.5:5	49.0	28.0	40.0
B. Human Plasmin + Bovine Fibrin				
Ribonuclease[3]	5	17.0	18.5	31.0
Plasmin[4]	50	86.0	80.0	77.5
Plasmin + Ribonuclease (10 : 1)	50:5	17.0	16.6	24.3
Plasmin + Ribonuclease (1 : 1)	5:5	21.0	19.3	23.7
Plasmin + Ribonuclease (1 : 10)	0.5:5	22.9	20.6	25.4
C. Trypsin	5	100.00	100.00	100.00
D. Pancreatin (NF)	5	100.00	100.00	100.00

[1]Bovine pancreatic ribonuclease - Armour and Co., Chicago, Illinois
[2]Bovine plasmin - Parke - Davis and Co., Detroit, Michigan
[3]Bovine ribonuclease - Wilson and Co., Chicago, Illinois
[4]Human plasmin (thrombolysin) - Merck Institute for Therapeutical Research, West Point, Pennsylvania

INHIBITION OF HYDROLYSIS OF FIBRIN BY PLASMIN USING RIBONUCLEASE

In another phase of the research program studies have been initiated to investigate the ability of ribonuclease to prevent the hydrolysis of fibrin by plasmin. The end point of such evaluation is the production of amino acids resulting from plasmin hydrolysis of fibrin. In this work, utilizing an all bovine system, plasmin is mixed with fibrin and the concentration of amino acids liberated by plasmin hydrolysis of fibrin in the presence and absence of ribonuclease is determined.

The methods of assay used and the results obtained to date are as follows:

A - Ribonuclease + Bovine Plasmin + Bovine Fibrin

To 0.7 cc of veronal buffer (0.0036 M) at the desired pH in a test tube are added 0.1 cc of bovine fibrinogen (5 mg/0.1 ml) in physiological saline, 0.1 ml of bovine plasmin (100γ/0.1 ml) in physiological saline and 0.1 ml of thrombin (100 NIH units/0.1 ml) in physiological saline. The final molarity of the versonal buffer is 0.0025. The contents of the tube are allowed to clot (approximately 30 seconds) and then the tubes are placed in a water bath at 37°C. The tubes are then observed until the fibrin cloth becomes completely liquified. At this t me, referred to as "lysis" time, 0.1 ml of a physiological saline solution of ribonuclease (1000γ/0.1 ml) is added to the tube. The contents of the tube are thoroughly mixed and 0.2 ml of the mixture is immediately withdrawn and assayed for total amino acids according to the method of Troll and Cannon (4) as modified by Taber (5) and adapted for our investigations. At intervals of 3 hours and 24 hours following the addition of the inhibitor 0.2 ml aliquots are removed from the tubes and assayed for total amino acid concentration.

The results of these investigations have been summarized in Figure II

B - Ribonuclease + Human Plasmin + Bovine Fibrin

For this phase of these studies certain modifications of the assay procedure described in Part A were made.
These are as follows:

1 - Use of phosphate buffer (0.03 MO systems for enzyme hydrolyses rather than barbituric acid buffers. The molarity and ionic strength of the phosphate buffer used were comparable to that of the Veronal buffer.

2 - Reduction of the concentration of bovine pancreatic ribonuclease utilized from 1000γ per 100γ of bovine plasmin to 500γ per 100γ with human plasmin. Investigations showed that the purer the ribonuclease used the ratio of ribonuclease to plasmin required for complete inhibition of the lysis of fibrin clots by plasmin decreased from 10:1 to 5:1.

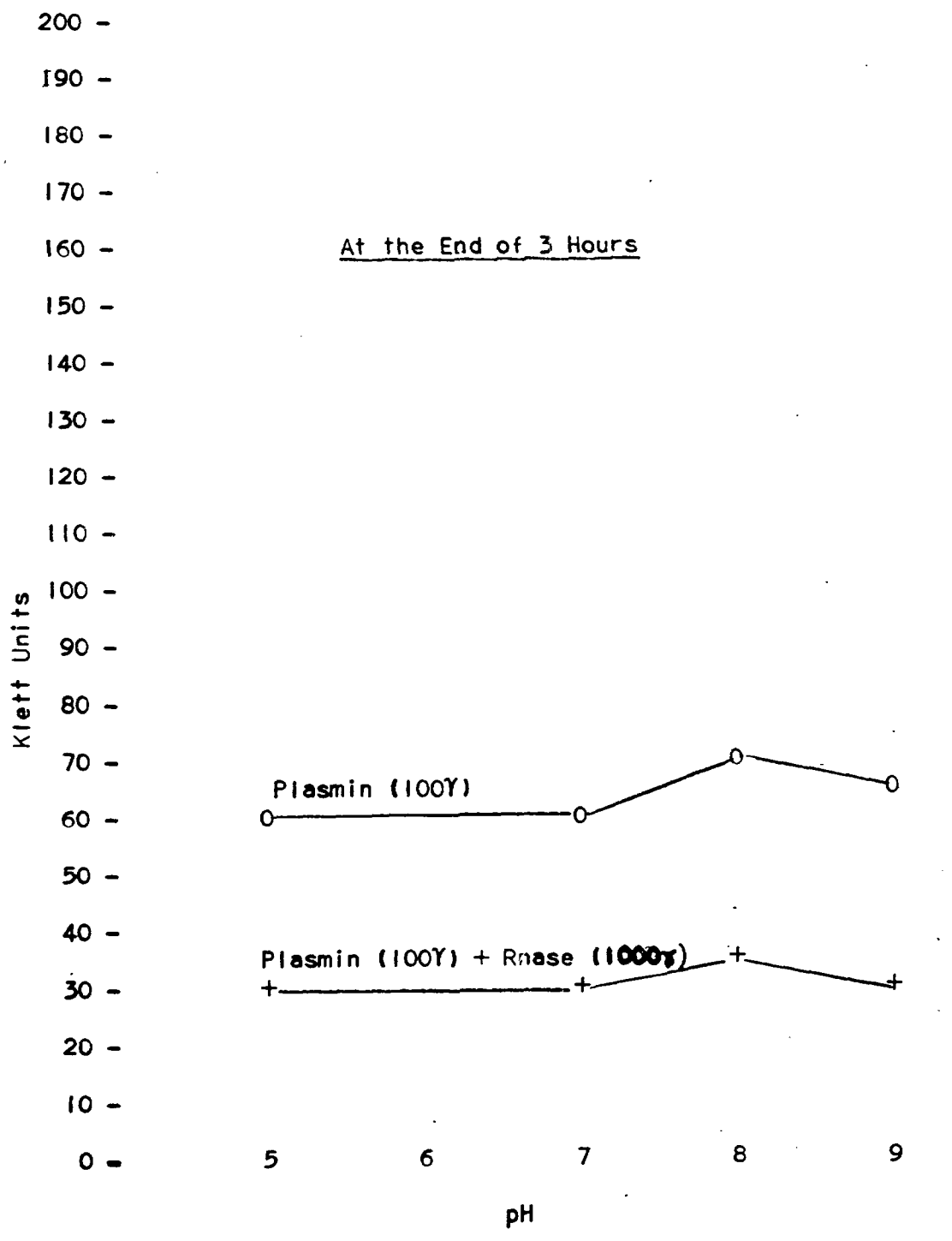
3 - Utilization of the Coleman Spectrophotometer rather than the Klett-Summerson Colorimeter.

All other conditions of assay remained constant as described in Part A.

The results obtained in work with human plasmin and ribonuclease are presented in Figure IIA.

FIGURE II

Total Amino Acid Production (in Klett Units) Resulting From The Hydrolysis of Bovine Fibrin by Bovine Plasmin at 37° C



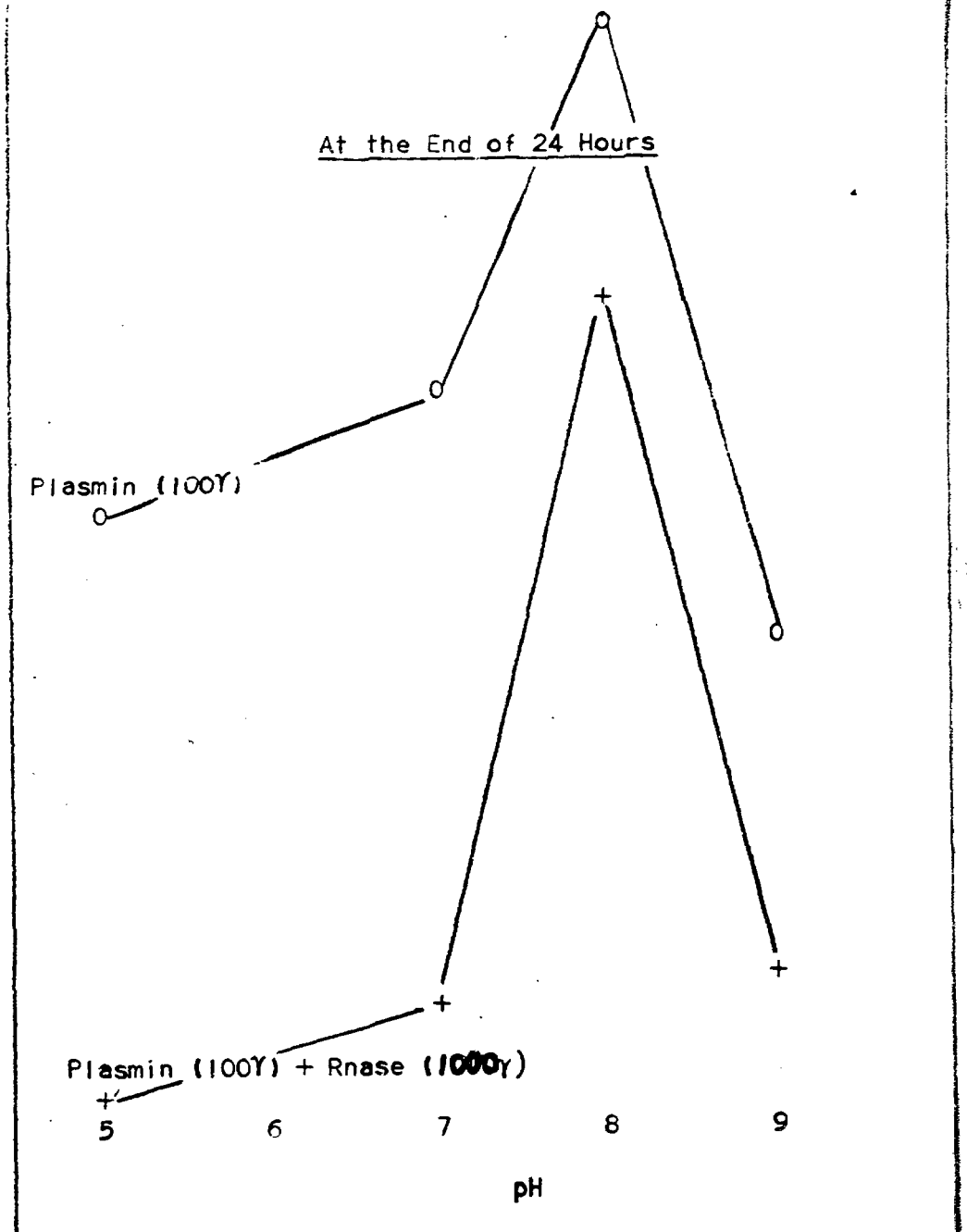
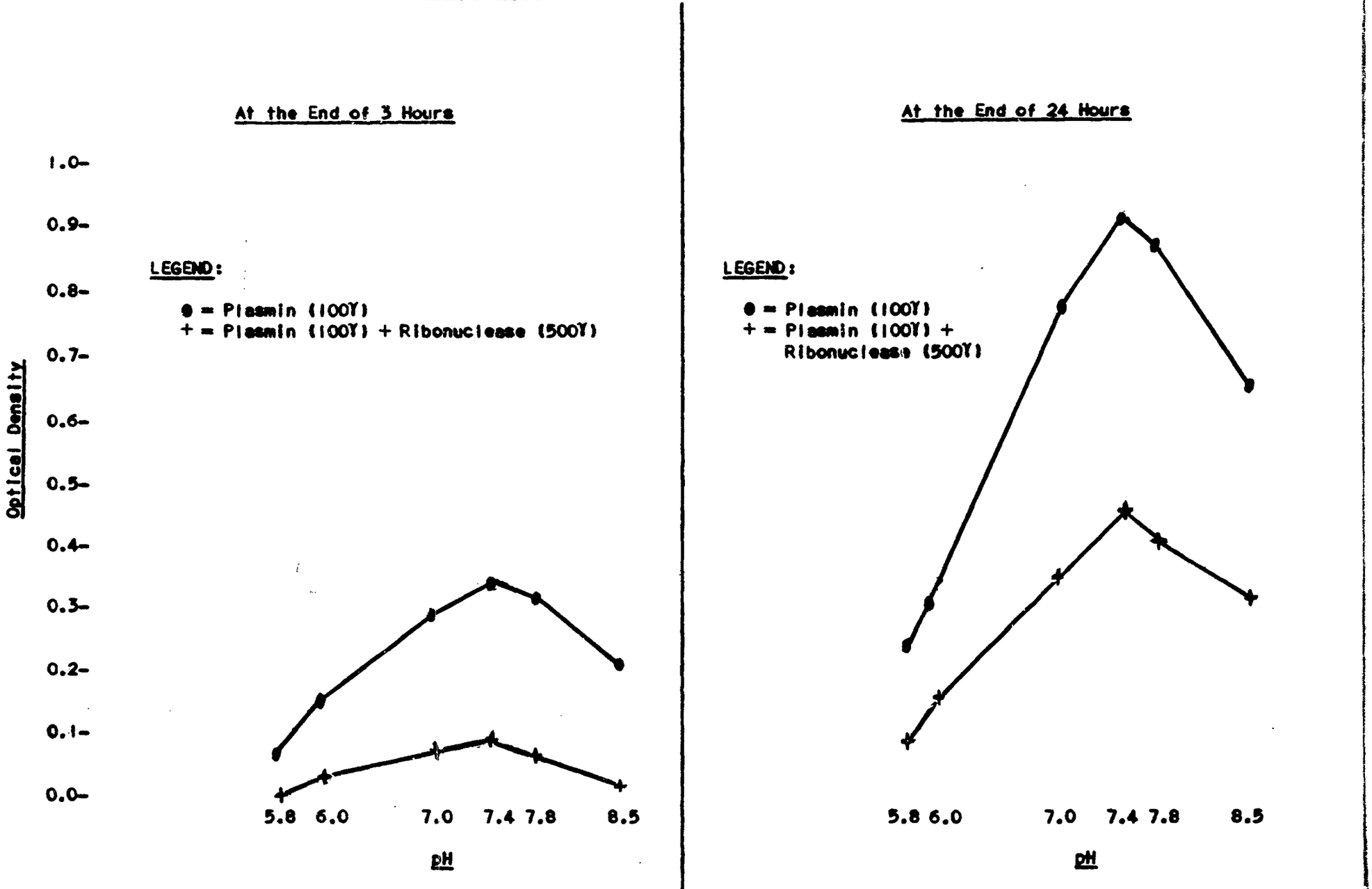


FIGURE IIA

Total Amino Acid Production Resulting From
The Hydrolysis of Bovine Fibrin by Human Plasmin



STUDIES ON THE ROLE OF HEPARIN AND RIBONUCLEASE IN FIBRINOLYSIS

Early studies made in our laboratories on the mechanism of fibrinolysis *in vitro* have shown bovine pancreatic ribonuclease to inhibit the depolymerization of bovine and human fibrin clots by either bovine or human plasmin (fibrinolysin).

Since heparin has been reported by many investigators to inhibit ribonuclease *in vitro* and *in vivo*, (heparin inhibition of cell division) and because of the use of ribonuclease for the determination of the heparin content of serum as reported by Lorenze (6), in 1960, we became interested in the relationship of heparin and ribonuclease in fibrinolysis. Our interest was further stimulated by the work of Gaertner and Liesiewicz (7), who tested the influence of ribonuclease on the activation of thrombin and the antithrombin efficacy of heparin. These investigators reported that pancreatic ribonuclease inhibited the specific antithrombin and anticoagulant activity of heparin, indicating a possible complementary reaction between ribonuclease and heparin in the clotting mechanism.

In view of our previous investigations of fibrinolysis and its inhibition by pancreatic ribonuclease and the reported role of ribonuclease and heparin in the clotting mechanism, we undertook a study of the heparin - ribonuclease interrelationship in fibrinolysis. This paper presents the results of these investigations.

Methods

All investigations were made in vitro utilizing bovine fibrin as a substrate for plasmin. Bovine fibrin clot assay system was prepared by dissolving in test tubes 5 mg. of bovine fibrinogen in 0.0275M saline phosphate buffer at the desired pH, adding 5 NIH units of bovine thrombin dissolved in 0.0275M saline phosphate buffer, and allowing the mixture of fibrinogen and thrombin to clot. The total volume of this assay system was 1.0 ml.

Our studies were carried out in two parts. In the first phase we investigated the role of heparin in effecting the lysis of bovine fibrin clots. In these studies heparin sodium was added to bovine fibrinogen in amounts from 1 to 1000 NIH units prior to the addition of thrombin. Immediately following the addition of heparin to bovine fibrinogen thrombin was added and the tubes allowed to stand until a clot formed (approximately one to five minutes). The tubes were then incubated in a water bath for 24 hours at 37.5°C until the fibrin clot became completely liquefied (lysed). The time of complete liquefaction was recorded as "lysis time." Partial liquefaction of a fibrin clot in 24 hours was considered an incomplete lysis and recorded as non-lysed fibrin. Any lysis or liquefaction occurring within 24 hours was considered significant. Assays were performed at pH's 7.0, 7.4, 7.8, and 9.0 in order to ascertain the influence of pH on the ability of heparin to induce lysis of a fibrin clot.

The results of these studies indicated that concentrations of one to ten units of heparin caused the liquefaction of the fibrin clots in our system at pH's 7.4 and 7.8. Lysis of the fibrin clots was not observed at any other concentration of heparin. Control clots, without heparin, did not autolyse or liquefy at any of the pH's studied.

Addition of 50 to 100γ of bovine ribonuclease inhibited the lytic activity of heparin obtained at pH 7.4 and pH 7.8. In this regard it is interesting to note that it required 100γ of ribonuclease to inhibit the lytic activity attributed to heparin at pH 7.8 and only 50γ of ribonuclease at pH 7.4. This is especially significant in view of the fact that the reported isoelectric pH of ribonuclease activity in depolymerizing nucleic acids.

Moreover, the addition of heparin to our test system did not interfere with the clotting of fibrinogen indicating, in accord with the concepts of many investigations, that the anticoagulatory activity of heparin is instrumental only in the prevention of the prothrombin-thrombin conversion.

The second phase of our experimental investigation was concerned with obtaining more specific information about the mechanism of the ribonuclease-heparin interrelationship in fibrinolysis. In these studies the effects or actions of heparin on plasmin and plasmin and ribonuclease were determined. Four different experiments were performed. In the first series, the effect of varying concentration of heparin on plasmin activity was ascertained. The second series of studies was made to determine if heparin could reverse the inhibition of plasmin activity by ribonuclease as previously observed in our laboratories. Experimental Series 3 and 4 were conducted to find out whether heparin's effects in the fibrinolytic system were via complexes with ribonuclease or plasmin.

In the third group or series of experiments varying concentrations of heparin were preincubated with a fixed concentration of ribonuclease, known to inhibit plasmin activity, prior to the addition of plasmin. In the final experiments plasmin was preincubated with varying concentrations of heparin prior to the addition of an inhibitory concentration of ribonuclease.

In all instances the fibrin clot assay described earlier was used as the test system with the time of complete liquefaction or lysis of the fibrin clot as the end point. All enzyme incubations were carried out at 37.5°C for 24 hours. As in initial studies made with heparin and fibrin, lysis occurring within 24 hours was considered significant. Preincubations of heparin and ribonuclease and heparin and plasmin were made at 37.5°C for 30 minutes before addition to the assay system. In all of these investigations 100 micrograms of human plasmin, activated by streptokinase, were used. In experiments with ribonuclease the concentration of this enzyme used was 50 micrograms.

Experiments in this part of our investigation were also conducted at pH's 7.0, 7.4, 7.8 and 9.0.

The results obtained in these studies have been summarized for each pH used and are presented in the attached tables.

Figure 3 shows the data obtained in studies made at pH 7.0. As can be noted, heparin exerts no effect on plasmin activity at any of the concentrations of heparin used. Lysis of fibrin clots in this system occurs within 30 to 60 minutes following incubation at 37.5°C. As can be noted in Curve No. 2, however, the addition of 50Y of ribonuclease inhibits lysis for six hours with a gradual increase in lysis time as the concentration of heparin increases. It is interesting to note that even at concentrations of 200 NIH units of heparin the inhibitory effect of ribonuclease is still evident. From the studies made in which ribonuclease was preincubated with heparin (Curve No. 3) it can be seen that low concentrations of heparin enhance the inhibitory activity of ribonuclease. Incubating heparin with plasmin at pH 7.0 prior to the addition of ribonuclease (Curve No. 4) decreases the fibrinolytic activity of plasmin possibly by the formation of a heparin-plasmin complex resulting in more effective inhibition of plasmin by ribonuclease.

Figure 4 presents the data obtained with studies made at the physiological pH of 7.4. As was obtained in the investigations made at pH 7.0 and in subsequent studies made at other pH's, heparin exerts no effect, inhibiting or activating, on plasmin activity (Curve No. 1). However, at this pH it requires more heparin to prevent ribonuclease from inhibiting plasmin activity (approximately 50 units as compared to 20 units at pH 7.0) as evidenced from examination of the curves. Preincubating heparin with ribonuclease results in complete removal of the inhibition of plasmin by ribonuclease (Curve No. 3), whereas preincubating heparin with plasmin allows for more effective ribonuclease inhibition of plasmin activity as found at Ph 7.0.

Figure 5 presents the data obtained with studies made at pH 7.8. In these studies data similar to that of other pH's was obtained with the noteworthy exception that ribonuclease activity as an inhibitor of plasmin is greatly

diminished. This could possibly be attributed to the fact that ribonuclease is at its isoelectric pH and consequently less effective because its net charge structure is neutral. Otherwise, the data is similar to that obtained at pH 7.4.

Data for studies made at pH 9.0 are given in Figure 6. In all instances data at this pH was similar to that obtained at pH's 7.4 and 7.8. However, with preincubation of heparin and plasmin, ribonuclease was found to be almost as effective in inhibiting plasmin activity with low concentrations of heparin present as was observed at pH 7.0. This again suggests the importance of molecular structure, especially in relation to "charge" distribution and patterns.

FIGURE 3

INTERRELATIONSHIP OF HEPARIN–RIBONUCLEASE–PLASMIN

AT pH 7.0

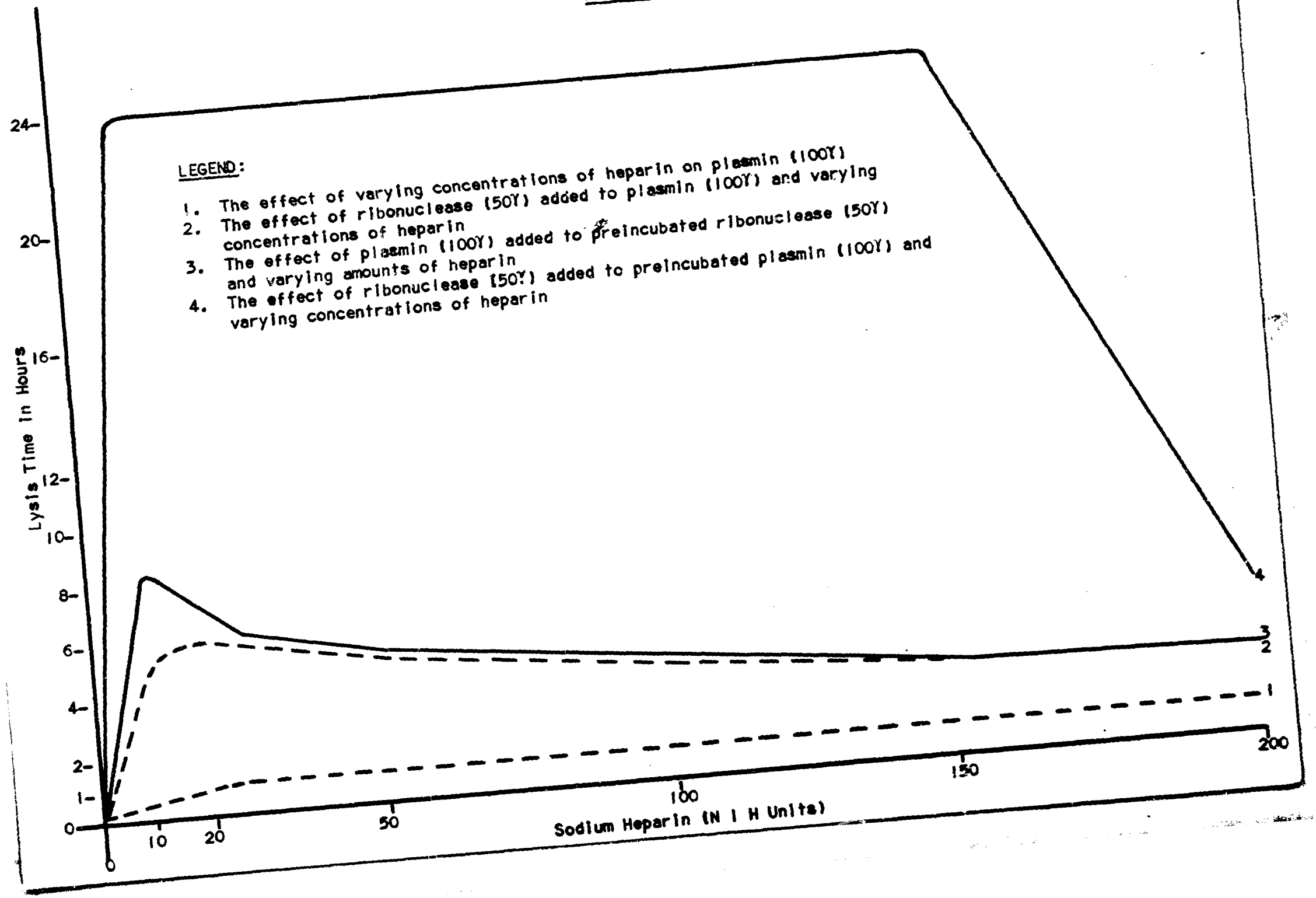


FIGURE 4

INTERRELATIONSHIP OF HEPARIN-RIBONUCLEASE-PLASMIN

AT pH 7.4

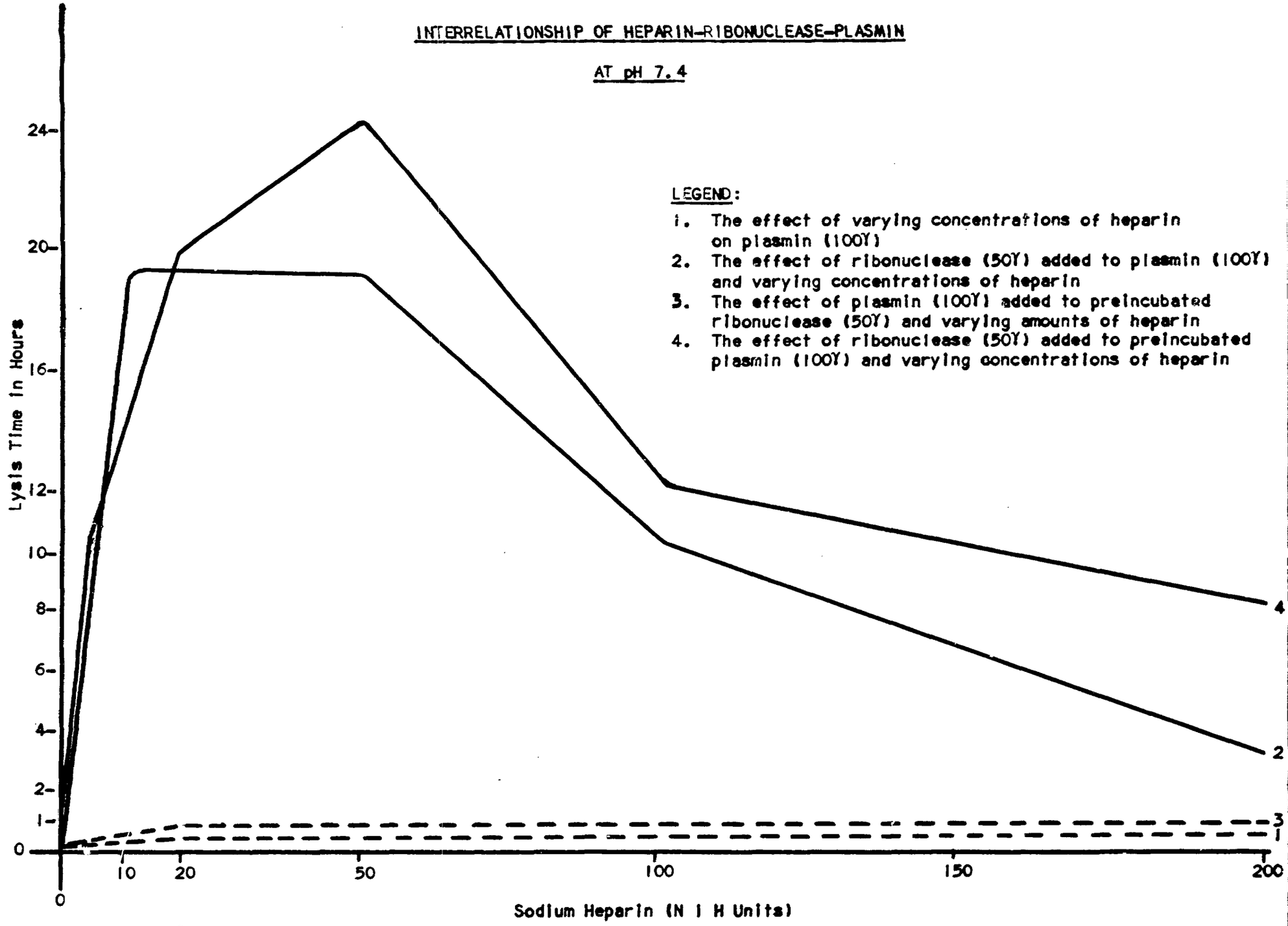


FIGURE 5

INTERRELATIONSHIP OF HEPARIN-RIBONUCLEASE-PLASMIN

AT pH 7.8

LEGEND:

1. The effect of varying concentrations of heparin on plasmin (100Y)
2. The effect of ribonuclease (50Y) added to plasmin (100Y) and varying concentrations of heparin
3. The effect of plasmin (100Y) added to preincubated ribonuclease (50Y) and varying amounts of heparin
4. The effect of ribonuclease (50Y) added to preincubated plasmin (100Y) and varying amounts of heparin

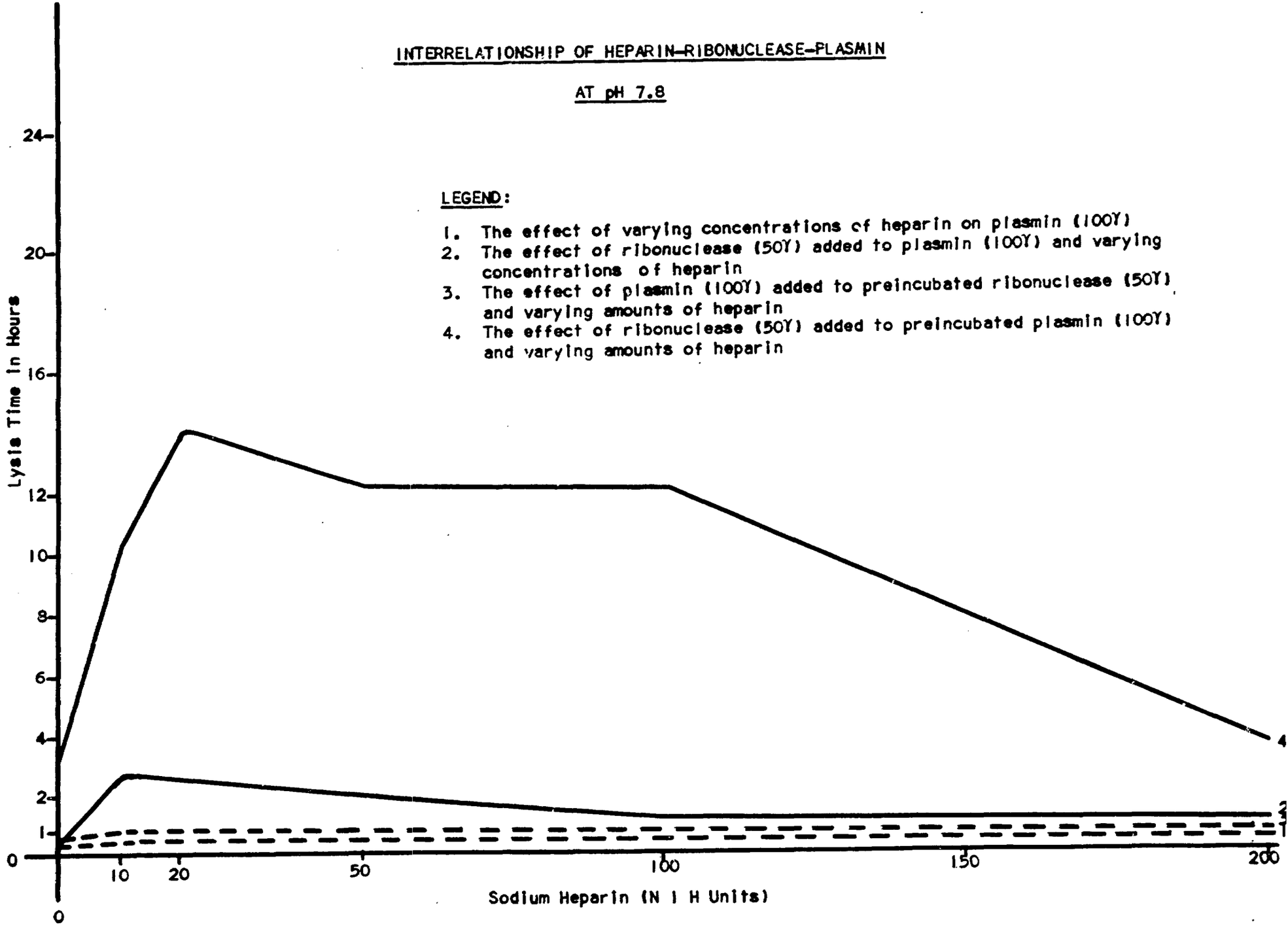
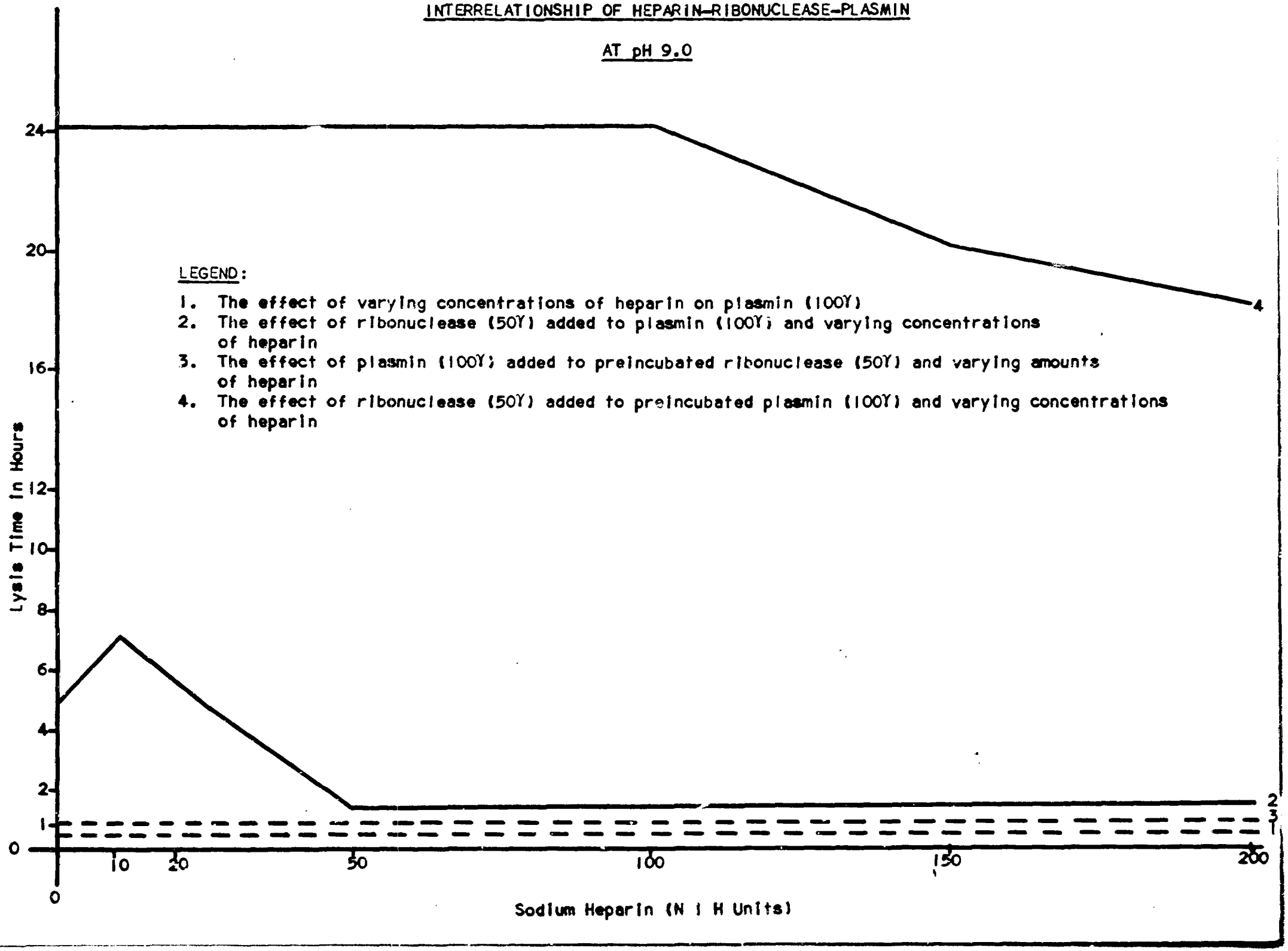


FIGURE 6

INTERRELATIONSHIP OF HEPARIN–RIBONUCLEASE–PLASMIN

AT pH 9.0



Investigation of Degraded Ribonuclease on Plasmin Activity

Investigations were initiated to ascertain if the antiplasmin activity of ribonuclease was a function of the intact ribonuclease or a specific amino acid grouping or active center in ribonuclease. Our approach in this part of our research was to determine the effects of mild acid hydrolysis on ribonuclease from both the point of view of its enzyme function in depolymerizing yeast nucleic acids and its antiplasmin activity.

Acid hydrolysis was accomplished by a modification of the method of Gordon, et. al. (8), who refluxed protein successfully with 6N hydrochloric acid up to 70 hours. We conducted the refluxing in the autoclave at 120°C and 15 pounds pressure.

In preliminary experiments 10 mg. quantities of bovine pancreatic ribonuclease were hydrolyzed in 10 ml. of 6N hydrochloric acid in sealed test tubes in the autoclave for periods of 20 minutes, 90 minutes, and 120 minutes. Following hydrolysis the hydrolysates were evaporated to dryness by boiling. The residue obtained was then diluted with 1 ml. of physiological saline to give a theoretical concentration of residue equivalent to 1000γ of ribonuclease per milliliter of saline. Aliquots of 0.1 (1000γ of hydrolyzed ribonuclease) and 0.2 (2000γ of hydrolyzed ribonuclease) ml. of the saline solution of the hydrolyzed ribonuclease were then assayed for antiplasmin activity using human plasmin (100γ) as the fibrinolytic enzyme. The method of assay for fibrinolytic activity was based on the time required for complete liquefaction of a fibrin clot, as described in our first progress report, using a phosphate buffer system.

The data obtained from these studies showed that all of the hydrolysates prepared and tested contained no antiplasmin activity.

Based on the work of Zollner, et. al. (9), who showed that hydrolysis of ribonuclease in the presence of heparin by boiling in 1/60N hydrochloric acid at 80°C for 30 minutes destroyed heparin but did not cause the loss of ribonuclease activity, we undertook a concentration study of the hydrochloric acid hydrolysis of ribonuclease. In these investigations 10 mg. amounts of bovine pancreatic ribonuclease were hydrolyzed by autoclaving for 15 minutes with varying concentrations of hydrochloric acid ranging from 5N to 0.01N. Following hydrolysis the hydrolysates were evaporated to dryness by boiling and dessicating under vacuum. The residue hydrolysates were then dissolved in physiological saline and assayed for antiplasmin activity at pH 7.4 using the fibrin clot lysis method. The residue hydrolysates were assayed for ribonuclease activity at pH's 5.0 and 7.4 by methods previously described and used in our research (Section A of this report).

The results of these studies are summarized in Tables VI and VII. Table VI presents the data obtained when the hydrolysates were assessed for antiplasmin activity. Table VII presents the data obtained when the hydrolysates were assayed for ribonuclease activity.

TABLE VI

Antiplasmin Activity of Hydrolysates of Acid Hydrolyzed Bovine Pancreatic Ribonuclease

Enzyme System	Hydrolysates From: 5N HCl	1N HCl	0.5N HCl	0.1N HCl	0.075N HCl	0.05N HCl	0.01N HCl	Control 100γ PI
	Time of Lysis in Minutes							
Human PI (100γ) + Hydrolysate (1000γ)	20	20	20	25	60	No Lysis	No Lysis	12
Human PI (100γ) + Hydrolysate (2000γ)	60	60	60	60	1200 (20 Hrs)	No Lysis	No Lysis	12

TABLE VII

Ribonuclease Activity of Hydrolysates of Acid Hydrolyzed Bovine Pancreatic Ribonuclease

Hydrolysates From	Concentration of Hydrolysate	% Transmission (260 Mμ) pH 5.0	pH 7.4
1.0N HCl	10γ	100	100
0.5N HCl	10γ	100	100
0.1N HCl	10γ	100	100
0.075N HCl	10γ	100	100
0.05N HCl	10γ	100	85
0.01N HCl	10γ	100	Not Determined
0.005N HCl	10γ	100	Not Determined
0.00N HCl	10γ	80	45
Ribonuclease Control-not autoclaved	10γ	30	23

The data from Table VI indicates that hydrolysis of ribonuclease in hydrochloric acid of more than 0.075 normality resulted in almost complete loss of the antiplasmin activity of the ribonuclease. This is of particular significance when considered together with the fact that regardless of the normality of hydrochloric acid used to hydrolyze ribonuclease the enzymatic activity of ribonuclease was destroyed (Table VII). It is interesting to note that when ribonuclease was autoclaved without any hydrochloric acid only 80% of its activity was lost; however, its ability to inhibit plasmin activity was retained although diminished.

In view of these observations the hydrolysates obtained in these investigations were examined chromatographically to determine the differences between hydrolysates in terms of amino acids liberated from the ribonuclease molecule. The hydrolysates were analyzed by paper chromatographic techniques using a Butanol-acetic acid-water (78-17-5) mobile phase. The components of the hydrolysates were allowed to separate for a period of 48 hours. At the end of this time the amino acids were developed using ninhydrin in butanol as the color-developing agent.

The results of these preliminary chromatographic separations revealed the presence of six distinct amino acids or peptide fractions in ribonuclease hydrolysates obtained with 1N and 5N hydrochloric acid. Only one amino acid or peptide fraction was obtained from hydrolysates prepared by autoclaving ribonuclease in 0.1N, 0.075N, 0.05N, 0.01N, or 0.005N hydrochloric acid.

This data strongly suggests that the loss of the antiplasmin activity of ribonuclease obtained with the 1N and 5N hydrochloric acid hydrolysis centers around the five as yet unidentified amino acids or peptide fractions obtained. Hydrolysis by the methods we used resulted in the loss of ribonuclease activity with apparent distinct formation of a single peptide or amino acid fraction.

From this data we have gained knowledge suggesting that the enzyme activity of ribonuclease and its antiplasmin activity are due to different centers or entities inherent in the molecule.

DISCUSSION AND SUMMARY

The ability of bovine ribonuclease to inhibit bovine and/or human plasmin activity has been demonstrated in vitro. It was observed in this regard that temperature and pH influence the nature and rate of enzyme-inhibitor substrate complex. As the temperature is increased the rate of enzyme degredation of a fibrin clot by plasmin increases. However in the presence of ribonuclease a shift in temperature function occurs and the optimal temperature for inhibition of plasmin activity by ribonuclease appears to be 37°C. Moreover it is noted that for both the plasmin and the plasmin + ribonuclease systems the pH for optimal enzyme activity appears to be pH 8. However inhibition of plasmin activity by ribonuclease is functional at all pH levels evaluated with complete inhibition being obtained at pH 8 when 500Y of ribonuclease is added to 100Y of plasmin.

The mode of action of ribonuclease inhibition of plasmin was studied and it was found that the inhibition of plasmin by ribonuclease appears to be primarily through the function of an enzyme (plasmin) ribonuclease complex in an apparent stoichimetric relationship.

Investigations of the inhibition of plasmin by E-amino caproic acid and Deoxyribonuclease indicate the ability of E-amino caproic acid to inhibit lysis of fibrin clots by ribonuclease whereas no inhibition of plasmin activity was exhibited by deoxyribonuclease at concentrations of deoxy-ribonuclease to plasmin as high as 1000:1.

In a separate investigation experiments were made to determine if other component biological substances of the fibrinolytic system possessed ribonuclease activity. In addition trypsin and pancreatin (NF) were also assessed for ribonuclease activity. The results of these studies (Table V) indicated that neither bovine plasmin, trypsin or pancreatin exhibit ribo-nuclease activity. Human plasmin did, however, evoke some depolymerization of yeast nucleic acid at all pH's evaluated. It was also observed that although ribonuclease will inhibit plasmin activity in the dissolution of a fibrin clot as much as 10 times more plasmin than ribonuclease does not inhibit ribonuclease activity significantly in depolymerzing yeast nucleic acid.

However it is interesting to note that at pH 5.0 and pH 8.6 there is a slight decrease in ribonuclease enzyme activity in the presence of plasmin.

Moreover the inhibition of ribonuclease by plasmin occurs at pH values on either side of the pH reported to be the isoelectric point for ribonuclease, as well as the optimum pH for ribonuclease activity, pH 7.7 - 7.8 (2). Of extreme interest in this regard is the fact that whereas the apparent optimum pH for ribonuclease inhibition of fibrinolysin is approximately pH 8.0 no inhibition of ribonuclease activity by plasmin is observed at this pH; consequently, suggesting that ribonuclease functions in the inhibition of fibrinolysis activity through a reaction between as specific molecular configuration inherent in ribonuclease and a specific active center on plasmin, with regard to the lack of ribonuclease activity in pancreatin. The fact

that pancreatin does not contain ribonuclease could be explained through two possibilities; namely, ribonuclease has been removed from the pancreatin in the processes used to prepare this product or the ribonuclease is bound to other components of pancreatin and is not available for assay by the procedure used to assess ribonuclease activity. The finding that pancreatin does not posses ribonuclease activity is especially significant when considered with the fact that pancreatin does have fibrinolytic activity that can be inhibited by ribonuclease. Trypsin the major protoelytic enzyme component of pancreatic juice and pancreatin has never been demonstrated to have ribonuclease activity, a fact confirmed in our work.

In addition examination of the data obtained in these studies indicated that plasmin of human origin does not possess ribonuclease activity in spite of the observation that human plasmin appears to cause some depolymerization of yeast nucleic acid. This can possibly be attributed to the fact that human plasmin is the activated form of plasminogen - the preparation used being activated with streptokinase - streptodornase (deoxyribonuclease) - and that the activator may have caused some depolymerization of ribonucleic acid. In further support of this contention is the observation made for both the bovine and human plasmin systems that the presence of plasmin apparently enhances the activity of ribonuclease, especially at pH's 7.8 and 8.6. The fact that such phenomena do not occur to any appreciable extent at pH 5.0 suggests the possibility that molecular charge distribution patterns are of extreme importance in the plasmin-ribonuclease complex.

At pH 7.8 ribonuclease is at its isoelectric point and also at the pH reported to be optimal for pancreatic ribonuclease enzyme activity. Our data confirms the latter concept. At pH's higher or lower than the ribonuclease isoelectric pH of 7.8 its activity as an enzyme decreases. The function of plasmin in promoting ribonuclease activity, however, appears to be greater at pH's above the isoelectric pH of 7.8 (8.6), suggesting a molecular combination of plasmin and ribonuclease through negatively charged bonds of a specific nature. This contention infers the presence of active centers similar in molecular configuration on either plasmin or ribonuclease or both required for inherent and purported enzyme activities as well as for the antiplasmin activity of ribonuclease. Moreover, it is interesting to note that the ratio of plasmin to ribonuclease is important in terms of ribonuclease activity. This is especially reflected in studies with human plasmin where it can be observed that apparently with reduction of the concentration of plasmin relative to the concentration of ribonuclease some inhibition of ribonuclease activity by plasmin is obtained. This data again suggests a specific molecular combination of plasmin and ribonuclease involving active centers for enzyme activity.

In the all-bovine system the effect of varying concentrations of bovine plasmin on ribonuclease activity is not as clear. The ratio of plasmin to ribonuclease necessary to inhibit plasmin activity is in the relationship of one to one and more effective at pH's 5.0 and 8.6. This again points out the role of electrostatic bonding between ribonuclease and plasmin.

Finally, the question of species specificity in relation to plasmin - ribonuclease interaction is in part answered through these investigations.

There appears to be no species specificity. Bovine and human plasmins are capable of interacting with bovine ribonuclease. Whether this phenomenon is characteristic of bovine and human plasmins can only be determined by additional studies using plasmin and ribonuclease from other animal species and other tissues.

Examination of the data in Figures II and IIA shows that bovine ribonuclease inhibits the hydrolysis of fibrin by bovine and/or human plasmins over a wide pH range.

However, the inhibitory effect of ribonuclease is minimized at pH 8.0, the isoelectric pH for ribonuclease but also the pH at which the lysis (depolymerization) of a fibrin clot by plasmin is most effectively prevented by ribonuclease. This would imply that plasmin has at least two active centers possibly involved in its ability to react with fibrin. One of these would be concerned with the depolymerization of a fibrin clot and the other with the subsequent hydrolysis of the depolymerized fibrin clot. These centers are most easily affected by changes in pH which would alter the charge distribution of the various components of the fibrinolytic system. This concept is supported by the fact that ribonuclease effectively inhibits hydrolysis of fibrin by plasmin at pH's of 7 or lower. Moreover, this is especially significant when it is considered with the observations made that plasmin will exert an inhibitory effect on ribonuclease activity at pH 5 and 9, more effective at pH 5 and pH 9, but not at pH 8.0; again upholding the concept of more than one active center for the plasmin - ribonuclease complex in terms of the activities of each enzyme. This we feel is of particular importance from the point of view of one enzyme inhibiting another enzyme by apparent non-enzymatic processes. That the relationship of plasmin to ribonuclease in fibrinolysis is rather specific and non-enzymatic is further supported by the observation that the ratio of ribonuclease to plasmin in preventing the dissolution of fibrin clot by plasmin has been found to be in the order of 5-10:1, and quite constant.

One additional observation of extreme importance is that obtained by comparing data at 3 hours with that of 24 hours. Most striking is the fact that the total amino acid concentration of the 24 hour samples of bovine plasmin + ribonuclease is significally lower at pH's of 5, 6, and 7 than that observed for the same system at 3 hours. No explanation is available at this time.

This finding is particularly significant when compared to the plasmin data which follows a predictable course in terms of hydrolysis, 24 hour concentrations of amino acids being significaly elevated over the levels obtained at 3 hours.

Examination of the data obtained in these studies (Figure IIA) reveal that bovine pancreatic ribonuclease inhibits the hydrolysis of fibrin by human plasmin over a wide pH range.

The one noteworthy difference between data obtained with human plasmin and that obtained with bovine plasmin is the pH at which optimal activity was obtained. With bovine plasmin the pH for optimal activity of plasmin appeared

to be 7.8 - 8.0, while that for human plasmin is 7.4.

It is also of interest to note in this respect that although ribonuclease inhibits human plasmin activity even in the presence of ribonuclease, optimal enzyme activity for plasmin is still found at pH 7.4. Similar results were obtained with bovine plasmin. This, in our opinion, is of extreme significance because the optimal pH for inhibition of the depolymerization of fibrin is approximately 7.8. One possible interpretation of such data is that there is more than one active center concerned in the function and activity of plasmin: one concerned with depolymerization of a fibrin clot and the other with hydrolysis of depolymerized fibrin.

Of equal importance in these investigations is the observation that there is an apparent lack of specificity with respect to the plasmin system used. Both bovine and human plasmin are inhibited by bovine pancreatic ribonuclease. Investigations with other ribonuleases would help resolve this area of interest.

If species specificity is not a factor in terms of the fibrinolytic system and its function in cardiovascular physiology, then a major breakthrough in the realm of the basic phenomenon of blood clotting is imminent.

In summary and conclusion we have observed that heparin in low concentration (one to ten units) induces liquefaction of fibrin clots at pH 7.4 and 7.8, which can be inhibited by ribonuclease. This observation indicates that heparin might be acting as an "enzyme" or may effect the dissolution of fibrin by molecular interaction. Further studies would have to be made to determine the exact mode of action of heparin on fibrin clots, especially in terms of destabilization vs. stabilization as has been purported by Csaba (10) to be the role of heparin in the sol-gel changes occurring in blood clotting.

Our studies also indicate that the antifibrinolytic activity of ribonuclease can be inhibited by heparin. The pH studies suggest that the inhibition of the effect of ribonuclease on plasmin by heparin revolves around molecular structure and charge configuration. At pH's on either side of the isoelectric point of ribonuclease, inhibition by heparin was more pronounced, although heparin still was effective in inhibiting the antifibrinolytic activity of ribonuclease at the isoelectric pH of ribonuclease, 7.8.

All components of our test system - fibrin, heparin, and ribonuclease - are highly charged molecules. Heparin's electronegative charge is the basis for anticoagulant action and the means of entering enzymes in the clotting mechanism. The possibility of heparin changing the electrocharges of the fibrin clot should also be considered. This electrical imbalance could be reversed by ribonuclease's positive charges present at pH's lower than the isoelectric pH, and its negative load at pH's more alkaline than the isoelectric pH, being of least force at the isoelectric point of ribonuclease.

Moreover, our data suggests that heparin enjoys a dual function in the fibrinolytic system: complexing with plasmin on the one hand and with

ribonuclease, and effective inhibitor of plasmin, on the other. This infers the possibility of a related structural configuration between plasmin, heparin, and ribonuclease. Further investigations will have to be undertaken to elucidate these findings.

Hydrolysis of ribonuclease by hydrochloric (1N and 5N) acid results in the liberation of six distinct amino acid or peptide fractions. The loss of these entities of the ribonuclease moiety results in a decrease in the ability of ribonuclease to inhibit plasmin and loss of ribonuclease enzyme activity.

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