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# ANNUAL PROGRESS REPORT

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"INVESTIGATION OF THE MECHANISM OF FIBRINOLYSIS"

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# ABSTRACT


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In *in vitro* studies bovine pancreatic ribonuclease has been found to inhibit the depolymerization and hydrolysis of bovine fibrin by plasmin of human origin. The human plasmin utilized was observed to enhance the nuclease enzyme activity of bovine pancreatic ribonuclease.

Studies made on the role of heparin and ribonuclease in fibrinolysis have indicated that heparin inhibits the antiplasmin activity of ribonuclease. In addition, it has been found that heparin in low concentration (1 - 10 NIH units) induces liquefaction of fibrin clots *in vitro* without added plasmin. Bovine pancreatic ribonuclease will inhibit this effect of heparin.

Dilute acid hydrolysis of bovine pancreatic ribonuclease (0.01N - 0.075N hydrochloric acid) results in the loss of the nuclease activity of ribonuclease but not the antiplasmin activity of ribonuclease.

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

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## INVESTIGATION OF THE MECHANISM OF FIBRINOLYSIS

### Introduction

During the past year investigations on the mechanism of fibrinolysis have been continued.

In order to gain further insight into the mechanism by which ribonuclease inhibits the lysis of fibrin clots, four separate studies were conducted. Two of these - investigations of the ribonuclease activities of the components of the fibrinolytic system and investigations on the inhibition by ribonuclease of the depolymerization and hydrolysis of fibrin by plasmin - were continued utilizing human plasmin as the fibrinolytic enzyme and bovine fibrin as the substrate. In previous work in these areas an all-bovine fibrinolytic system had been employed.

Studies were also initiated to determine whether or not the ribonuclease molecule is required *in toto* for antiplasmin activity. In this work ribonuclease is being degraded chemically and enzymatically into peptides and amino acid fractions. These components are being assessed for antiplasmin activity.

In the final series of studies made during the period covered by this report, experiments were conducted 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.

This report presents the methods, data, and results obtained in the aforementioned studies during the period from July, 1962 through June, 1963.

A. Investigation of the Ribonuclease Activity of the Fibrinolytic System

Studies were continued to determine if the various biological materials comprising the fibrinolytic system used in our work possessed ribonuclease activity or inhibited the enzymatic function of ribonuclease. Evaluations of ribonuclease activity were carried out according to the methods of Anfinsen, et. al. (1), as described by Glick (2). Details of this method and the analytical procedure used in our work are presented in our Annual Progress Report, November 1, 1961 through June 30, 1962, previously submitted.

Systems studied include bovine plasmin, bovine pancreatic ribonuclease, bovine plasmin plus bovine ribonuclease, human plasmin, and human plasmin plus bovine ribonuclease. No additional studies were made with pancreatin or trypsin since earlier work (previously reported) indicated that these enzymes do not possess ribonuclease activity. They are capable, however, of hydrolyzing ribonuclease and are to be used for this purpose in other studies.

Determination of ribonuclease activity was made at pH's 5.0, 7.8, and 8.6.

The results of the investigations of the ribonuclease activity of the components of the fibrinolytic system employed in our research efforts are summarized in the following table.

TABLE I

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 |

[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

Examination of the data obtained in these studies indicates 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 point 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 andhuman plasmins are capable of interacting with bovine ribonuclease. Whether this phenomenon is characteristic of bovine and human plasminscan only be determined by additional studies using plasmin and ribonuclease from other animal species and other tissues. Such investigations are planned as part of our continuing effort.

B. Inhibition by Ribonuclease of the Depolymerization and Hydrolysis of Fibrin by Plasmin

Studies were continued in ascertaining the role of ribonuclease in preventing the depolymerization and subsequent hydrolysis of fibrin by plasmin. In earlier work these investigations were made utilizing bovine plasmin. In our current program of research on this problem, as presented in this report, we have been and are continuing the investigation of the antifibrinolytic activity utilizing human plasmin as our fibrinolytic enzyme and bovine pancreatic ribonuclease as the source of antifibrinolysin.

Although the parameters of observation and measurement observed in studies with human plasmin were the same as presented in our last progress report for studies made with bovine plasmin - namely, the production and identification of amino acids resulting from plasmin hydrolysis of fibrin - we have modified our assay procedures in order to achieve a more physiological system. The modification consisted of employing a phosphate buffer (0.03M) system for carrying out enzyme hydrolyses rather than the previously used barbituric acid buffer. The molarity and ionic strength of the phosphate buffer used was comparable to that of the Veronal buffer.

A second modification used was the reduction of the concentration of bovine pancreatic ribonuclease used as a constant concentration of inhibitor from 1000γ/100γ of plasmin to 500γ/100γ of plasmin. This modification was used primarily because of the fact that a change in our source

of supply of ribonuclease was necessitated due to a discontinuance of production of this product by the manufacturer. A new source of ribonuclease was obtained and assay of this material revealed it to be of a higher quality and purity than that previously used. Investigations in our laboratory showed that with the purer ribonuclease the ratio of ribonuclease necessary for complete inhibition of plasmin in vitro dropped from 10:1 to 5:1. This observation in itself augments the concept of the highly specific function of ribonuclease in the fibrinolytic system.

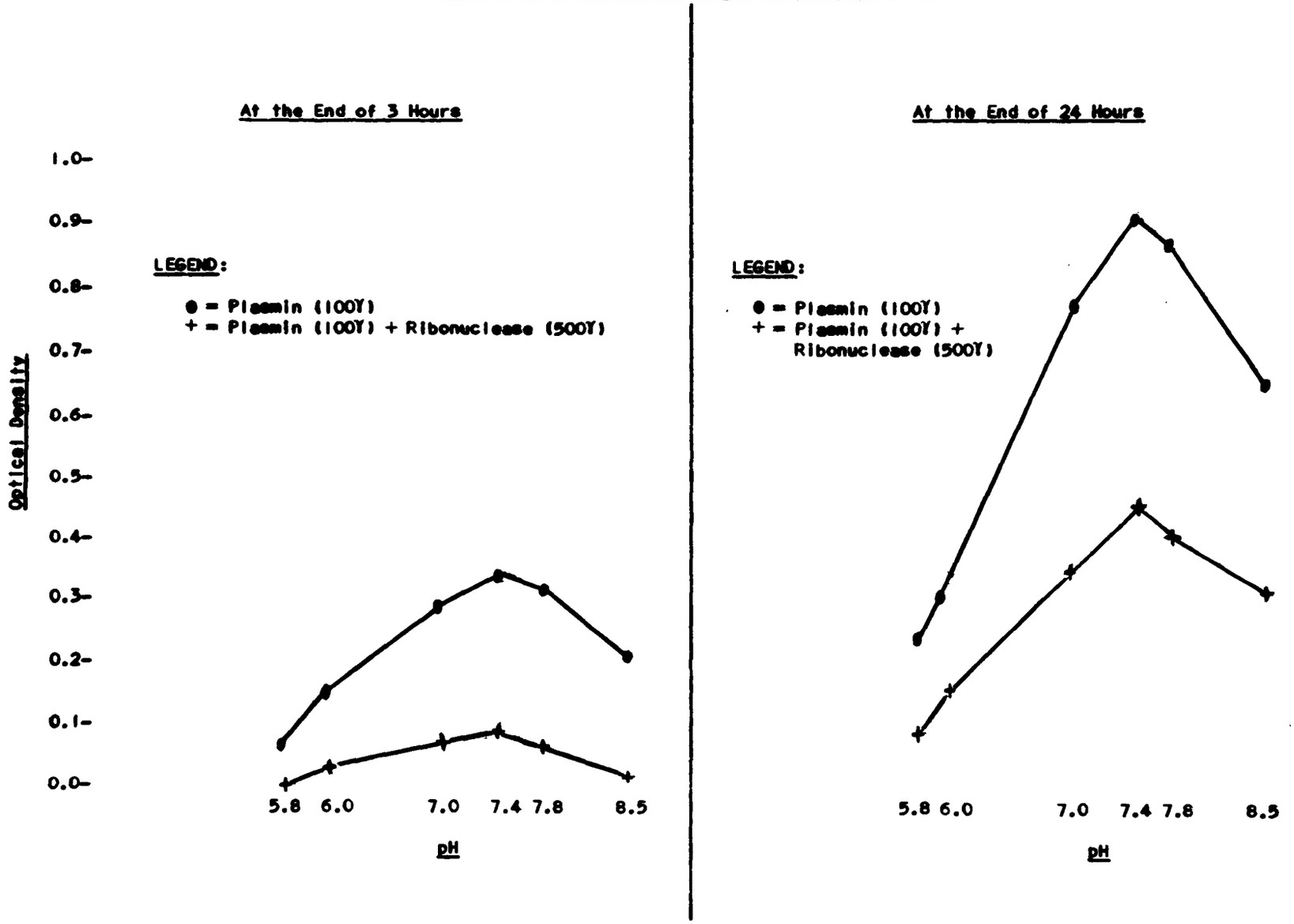
The third and final modification employed was the recording of changes in amino acid formation by recording changes in optical density as measured using the Coleman Spectrophotometer rather than the Klett-Summerson Colorimeter.

All other procedural aspects of these studies were identical to those reported in our prior progress report covering the period from November 1, 1961 through June 30, 1962.

The results of these investigations have been summarized in Figure 1.

FIGURE 1

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



## Discussion

Examination of the data obtained in these studies (Figure 1) reveal that bovine pancreatic ribonuclease inhibits the hydrolysis of fibrin by human plasmin over a wide pH range. Similar results were obtained with bovine plasmin.

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 ribonucleases 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.

Work is being continued in this part of our research effort in order to obtain more information as to the mechanism of fibrinolysis. Studies are in process by which we will ascertain the effects of the variation of the concentration of ribonuclease in preventing the hydrolysis of fibrin by plasmin. The effects of temperature are also being investigated.

Planned for the near future and in conjunction with work now in process is an examination of the end products (amino acids) produced by plasmin hydrolysis of fibrin in the presence and absence of ribonuclease. Some investigations have been carried out but are too preliminary in nature to report at this time with one noteworthy exception: whenever ribonuclease is incorporated into a fibrinolytic system of plasmin and fibrin we have noted, after chromatographic examination of the hydrolysate, the absence of several amino acids and/or peptides. These have not been identified to date.

C. Investigation of Degraded Ribonuclease on Plasmin Activity

During the past year investigations were initiated to ascertain if the antiplasmin activity of ribonuclease is a function of the intact ribonuclease or a specific amino acid grouping or active center in ribonuclease. Our first approach in this part of our research has been 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. (3), 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 of 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 (100γ of hydrolyzed ribonuclease) and 0.2 (200γ 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 hydrolsates prepared and tested contained no antiplasmin activity.

Based on the work of Zollner, et.al. (4), 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 II and III. Table II presents the data obtained when the hydrolysates were assessed for antiplasmin activity. Table III presents the data obtained when the hydrolysates were assayed for ribonuclease activity.

TABLE II

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 100Y PI |
|---|---|---|---|---|---|---|---|---|
| | Time of Lysis in Minutes | | | | | | | |
| Human PI (100Y) + Hydrolysate (1000Y) | 20 | 20 | 20 | 25 | 60 | No Lysis | No Lysis | 12 |
| Human PI (100Y) + Hydrolysate (2000Y) | 60 | 60 | 60 | 60 | 1200 (20 Hrs) | No Lysis | No Lysis | 12 |

TABLE III

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 | 10Y | 100 | 100 |
| 0.5N HCl | 10Y | 100 | 100 |
| 0.1N HCl | 10Y | 100 | 100 |
| 0.075N HCl | 10Y | 100 | 100 |
| 0.05N HCl | 10Y | 100 | 85 |
| 0.01N HCl | 10Y | 100 | Not Determined |
| 0.005N HCl | 10Y | 100 | Not Determined |
| 0.00N HCl | 10Y | 80 | 45 |
| Ribonuclease Control- not autoclaved | 10Y | 30 | 23 |

The data from Table II 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 III). 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 the 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. Investigations are now in process to elucidate the nature and type of centers and inherent amino acid molecules involved.

D. 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 or 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 (5) 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 Lisiewics (6), 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 autolyze 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 is 7.8 - also the pH reported to be optimal for 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 concentrations 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 2 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 3 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. Pre-incubating heparin with ribonuclease results in complete removal of the inhibition of plasmin by ribonuclease (Curve No. 3), whereas preincu-bating heparin with plasmin allows for more effective ribonuclease inhibition of plasmin activity as found at pH 7.0.

Figure 4 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 5. 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.

## Summary and Conclusions

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 (7) 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 thought to enter enzymes in the clotting mechanism. The possibility of heparin changing the electro-charges 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, an 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.

FIG 2

INTERRELATIONSHIP OF HEPARIN–RIBONUCLEASE–PLASMIN

AT pH 7.0

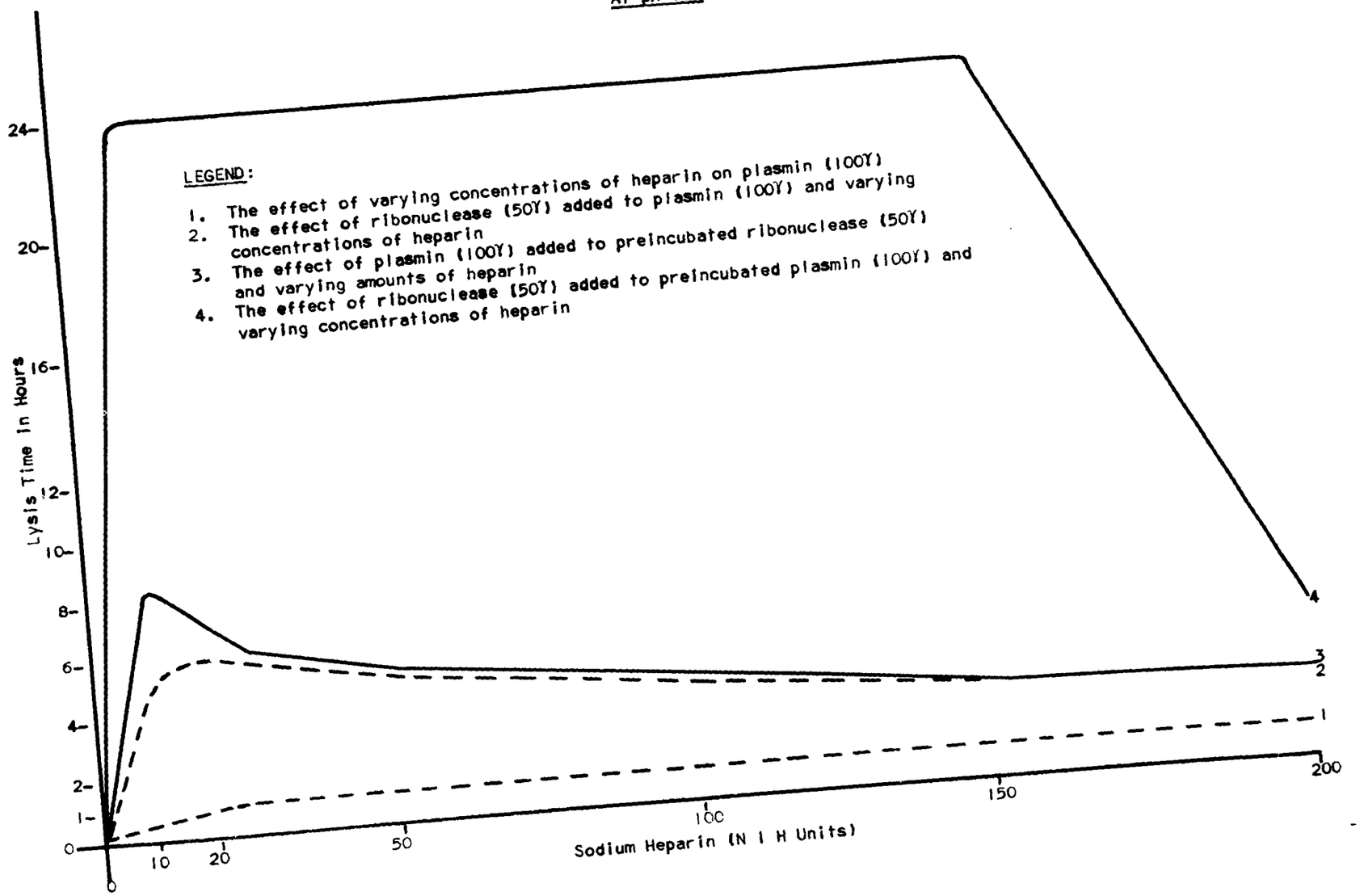
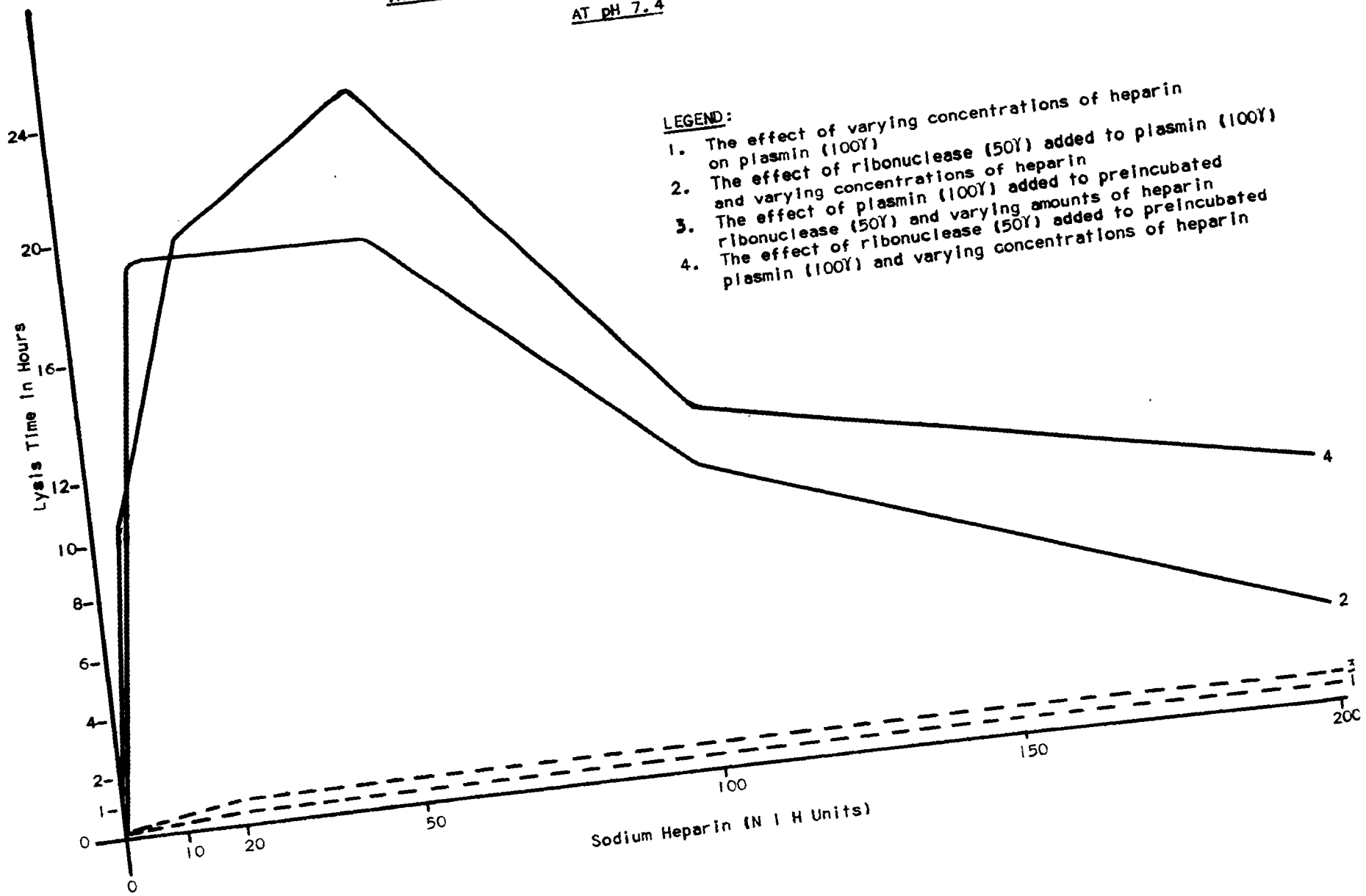


FIGURE 3

INTERRELATIONSHIP OF HEPARIN-RIBONUCLEASE-PLASMIN

AT pH 7.4



**FIGURE**

INTERRELATIONSHIP OF HEPARIN–RIBONUCLEASE–PLASMIN

AT pH 7.8

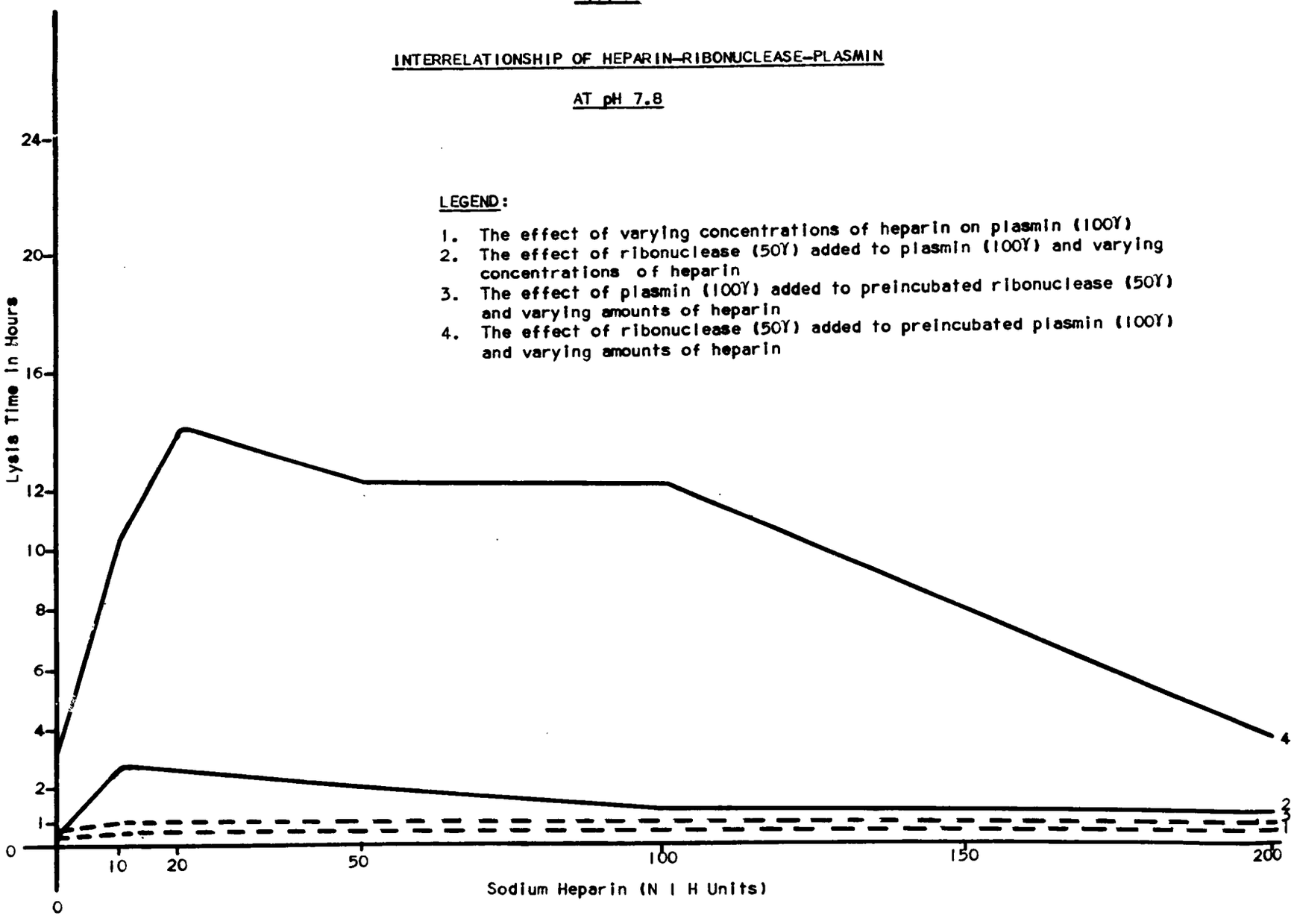
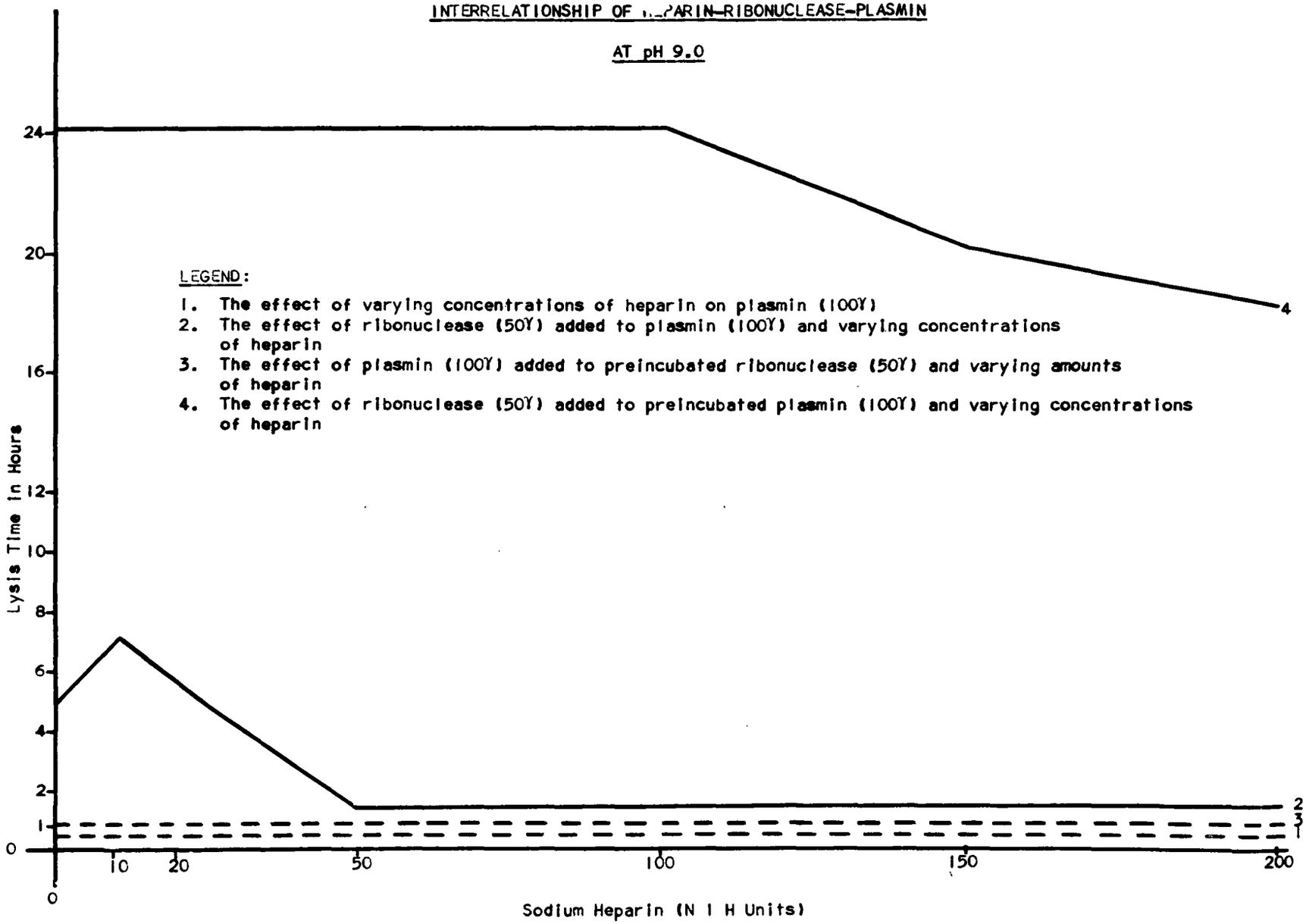


**FIGURE 5**

INTERRELATIONSHIP OF HEPARIN-RIBONUCLEASE-PLASMIN

AT pH 9.0



## SUMMARY

1. In *in vitro* studies human plasmin has been shown to be devoid of nuclease activity *per se*. It has been found that both bovine and human plasmin apparently enhance the nuclease activity of bovine pancreatic ribonuclease.
2. The ability of human plasmin to depolymerize and hydrolyze fibrin has been found to be inhibited by bovine pancreatic ribonuclease *in vitro*. Similar results were obtained with bovine plasmin.
3. Acid hydrolysis of ribonuclease results in the loss of ribonuclease activity at all hydrolysis levels from 0.005N to 5N hydrochloric acid used. No loss of the antiplasmin activity of bovine pancreatic ribonuclease was observed following autoclaving of ribonuclease at 120°C and 15 pounds pressure for 15 minutes in 0.01N to 0.075N hydrochloric acid. Chromatographic examination of the 0.01N – 0.075N hydrochloric acid hydrolysates revealed the presence of an unknown peptide or amino acid fraction. Paper chromatography of hydrolysates of ribonuclease obtained from autoclaving bovine pancreatic ribonuclease in 0.1N to 5.0N hydrochloric acid indicates the presence of six unknown amino acid or peptide fractions.
4. Heparin in low concentration (1 to 10 NIH units) induces liquefaction of fibrin clots sans plasmin at pH 7.4 and 7.8. Bovine pancreatic ribonuclease will inhibit this effect of heparin.

   Heparin inhibits the antiplasmin activity of ribonuclease. This effect of heparin has been observed to be more pronounced at pH levels above or below the ribonuclease isoelectric pH of 7.8.

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