HISTAMINE AND OTHER COMPOUNDS IN THE ACTIVATION OF PURIFIED PROTHROMBIN

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Marvin Murray, M. D.

1956

This is to certify that the

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Histamine and Other Compounds in the

Activation of Purified Prothrombin

presented by

Marvin Murray

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Physiology

Lester F Wolterink

Major professor

Date November 8, 1956

HISTAMINE AND OTHER COMPOUNDS IN THE ACTIVATION OF PURIFIED PROTHROMBIN

by

MARVIN MURRAY, M. D.

ABSTRACT OF A THESIS

Submitted to the School for Advanced Graduate Studies
Michigan State University of Agriculture and
Applied Science in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology

1956

ABSTRACT

The chemical reactions that occur when thrombin is formed from prothrombin are not fully understood. Attempts are being made to elucidate this complex problem by isolating and studying systems containing purified prothrombin. Efforts are also being made to find substances that activate prothrombin in purified form.

When prothrombin preparations containing small amounts of Ac-globulin are mixed with calcium, platelet extracts and a partially purified platelet co-factor, thrombin forms rapidly. The platelet co-factor obtained from plasma is considered to be the antihemophilic factor. In the work of this thesis, certain synthetic organic compounds were discovered which will substitute the platelet co-factor in the activation of purified prothrombin. If there are other known instances where an organic compound will substitute for a protein they must be rare. These findings also have implications related to hemophilia problem on the basis that research for therapeutic agents is encouraged. Moreover, technical difficulties are greatly reduced for studying the role of the platelet derivatives in the activation of prothrombin.

Histamine and its analogues, Benadryl, Decapryn and Linadryl (dimethylaminoethoxy compounds) and Phenindamine (a pyridindene derivative) were studied in detail. It was shown that their activity is in conjunction with that of platelet extract and plasma Ac-globulin. Moreover, the chemically active portion of the antihistamines has been identified. Acceleration of this activation was accomplished by the addition of as little as four units of purified thrombin to the activation mixture. This purified thrombin was obtained from purified prothrombin by activating in 25%

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sodium citrate solution. If thrombin is not added to the activation mixture, a full yield of thrombin from the prothrombin substrate may not result. It seems likely here that thrombin is functioning in its well known effect of converting plasma Ac-globulin to serum Ac-globulin which in turn accelerates the conversion of prothrombin.

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I wish to express my gratitude and deep sense of appreciation to Dr. Walter H. Seegers for his interest and assistance, without which this work could never have been done. I particularly wish to thank him for opening the facilities of his laboratory to me and for his patience and guidance as a teacher. My thanks are also expressed to Dr. S. A. Johnson for her assistance and ability to teach the techniques of coagulation research. I also wish to thank all the members of the Department of Physiology and Pharmacology of Wayne University, College of Medicine for their assistance and encouragement.

I add my gratitude to Dr. Lester Wolterink for his encouragement and his ability as a mentor of scientific thought. Many thanks belong to Dr. B. V. Alfredson and all the members of the Department of Physiology and Pharmacology at Michigan State University for their aid and encouragement.

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INTRODUCTION AND LITERATURE REVIEW

For many years, observations from clinical and conventional laboratories have been reported implicating histamine and other compounds with blood clotting reactions. Although many of these reports are conflicting, and the total amount of actual evidence is meager, reports from so many investigators indicate that a poorly defined relationship exists between these compounds and the clotting mechanism. In my series of experiments an attempt has been made to demonstrate that histamine and various analogues, a series of antihistaminic compounds, act as thromboplastic agents.

The following paragraphs give only a partial review of compounds implicated in clotting and in therapy of hemophilia (a defect in the first stage of coagulation).

Page and co-workers[1] reported that intravenous oxalic acid reduced coagulation time to normal within three days. This was refuted by Foster[2] who showed that high doses of oxalic acid delayed coagulation times (in rabbits).

Macht in studies of the effects of commonly used drugs on the coagulation mechanisms stated that mercuhydrin, mercupurin and thiomerin[3] decreased coagulation times of normal individuals, but theophylline had no effect. Similarly, he stated that digitalis glucosides[4] have a thromboplastic effect and decrease coagulation time.

Estrogenic compounds[5] have been used in the treatment of hemophilia and their efficacy has been shown by others[6] to be nil.

In 1951 Dunn and Lyons[7] reported that the diethylamine and diethanolamine salts of 3,5 diodo- 4 - pyridone N acetic acid decreased the

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number of hemorrhagic diatheses of hemophilic patients.

Another most interesting group of compounds is that containing the amino acids, histamine and the anti-histamines. Hecht[8] stated that glutamic acid and serine inhibited the first stage of the clotting reaction. de Vries, Schwager and Katchalski,[9] in an excellent series of experiments, demonstrated that poly L lysine (a basic polyamino acid) and protamine were antithrombic in action and were therefore anticoagulants in whole blood. However, both were antiheparins and could be neutralized by acidic polyamino acids.

Some of the earliest experiments relating the amines to the coagulation mechanisms can be traced to the reactions of foreign proteins in animals. It is a well known phenomenon that the blood becomes incoagulable in anaphylactic shock. This is apparently due to the release of more circulating heparin into the blood stream. A similar effect can be produced in histamine shock and peptone shock. The heparin can then be titrated with protamine or toluidine blue.

In the case of irradiation some investigators have indicated that plasma thromboplastin is decreased.[10] However, the consensus of opinion is that the primary factor responsible for decreased coagulability of the blood following whole body irradiation is due to a thrombocytopenia.[11]

In 1936, Temperly et al[12] prepared a denatured form of egg white protein which they claimed decreased coagulation times in non hemophiliacs and hemophiliacs. They also claimed remarkable palliation of the disease. Similarly, Castex and Pavlowsky[13] stated that acetone extracts of cream and milk decreased the coagulation times of both normal and hemophiliac blood.

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Further knowledge of the chemistry of the intrinsic agents of the clotting mechanism gave new insight into the action of thromboplastic agents. Trypsin can activate prothrombin. In experiments with humans, Tagnon[14] reported that intravenous trypsin reduced coagulation times in hemophilia.

There have been many reports stating that histamine will shorten the coagulation time of the blood in various animals. Osterieth and Le-Comte[15] reported that intravenous histamine in doses of 1-50 mg/Kg would shorten the coagulation time of blood taken from rabbits shortly after its administration. In dogs, following massive doses of histamine given intravenously brain lesions are observed similar to those seen when thromboplastin is given intravenously.[16]

In a study by Valentine and Lawrence,[17] it was found that (a) plasma histamine concentration is from 0-5% of that in whole blood. (b) normal serum histamine concentration is from 20-50% of that in whole blood and (c) in leukemic patients serum histamine concentration is from 0-5% of that in whole blood. These data are particularly interesting from the standpoint that many patients with leukemia have clotting deficiencies despite platelet concentrations which are within the normal range.

With respect to the subject under consideration, reports on the anti-histamines are as yet meager and conflicting. Zeller[18] reported that histamine reduced the coagulation time in rabbits and that Anthistine and Neo-antergan blocked this effect as well as prolonging coagulation times in control animals. Haley[19], on the other hand, found a definite but small decrease in the blood coagulation times in normal and heparinized rabbit plasma which had been treated with histamine and anti-histaminics.

In a series of experiments involving hemophilic children, Butler and Sandford et al[20] found that histamine given intravenously in doses of

0.2 to 2 mgm. of histamine base was followed by a decrease in the coagulation time of whole blood. After further experiments, they stated that decreased coagulation time of whole blood was associated with an increase in platelet fragility and a consequent increase in platelet disintegration.[21]

Another interpretation of the action of histamine has been given by Gerendas et al.[23] It is their contention that heparin increases the rate of inactivation of thrombin, whereas histamine decreases thrombin inactivation and in this way decreases the coagulation time. They stated that this is a physiologic mechanism and that an equilibrium exists between heparin and histamine.

With the earliest work in defining the blood coagulation reactions, both Morawitz and Howell presented the schemes which outlined these reactions. These may be noted as follows:

1. Prothrombin $\xrightarrow[\text{calcium}]{\text{thromboplastin}}$ Thrombin

2. Fibrinogen $\xrightarrow{\text{thrombin}}$ Fibrin

Since those early days, a much more detailed concept of the coagulation mechanism exists; yet even today, the experiments and factors involved in the coagulation mechanism have not been fully outlined. We must still suffice with the working schemes which include the many observed factors which we recognize in the clotting system. For the purpose of these experiments we shall consider the system on the Frontispiece as outlined by Seegers.[24]

Of all the compounds in the coagulation mechanism, only two are relatively well known. The first, prothrombin, which is the basic compound involved in the first stage of the coagulation mechanism, and the second, fibrinogen, the central compound in the second stage of the reaction. In

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these experiments we will be concerned mainly with the first stage of the coagulation mechanisms, which by definition involves the conversion of prothrombin to thrombin. From the results of the work and the purification of prothrombin it is established that prothrombin is the substrate from which thrombin activity arises. This has been particularly well established by the experiments showing the auto-catalytic activation of prothrombin in 25 percent sodium citrate solution.[25] In these experiments it was found that only prothrombin contains the necessary material from which thrombin could be derived. The advantage of having a highly purified reagent with which to work in delineating and elucidating the first stage reaction are occasionally nullified by the fact that other compounds associated with the conversion of prothrombin to thrombin are much less than pure. The chemical nature of the first stage reaction remains then to be elucidated. At present, to purified prothrombin singly, partially purified activators and inhibitors can be added and their effects studied. With the exception of heparin, it seems likely though that no activator or inhibitor now available is in as a purified a form as prothrombin itself.

Many substances have been described in the literature but only a few of them are required for rapid prothrombin activation and a full yield of thrombin, a point in fact, Ac-globulin, platelet extract and a platelet co-factor of the plasma, together with calcium seem to be quite adequate for the rapid production of thrombin. This is indicated as follows:

3. Prothrombin $\xrightarrow[\text{Ac-globulin, Platelet extract, Platelet co-factor}]{\text{calcium}}$ Thrombin

In these reactions, plasma Ac-globulin is considered to be changed to serum Ac-globulin in the presence of small amounts of thrombin.[26]

Eventually, serum Ac-globulin changes to inactive Ac-globulin:

4. Plasma Ac-globulin $\xrightarrow{\text{thrombin}}$ Serum Ac-globulin

5. Serum Ac-globulin $\xrightarrow{\text{thrombin}}$ Inactive Serum Ac-globulin

One of the activities of platelet functions with plasma a co-factor and is almost equivalent to true thromboplastin. That is to say, in the presence of Ac-globulin, calcium, platelets and the platelet co-factor from plasma, purified prothrombin changes to thrombin.[27] With the purified prothrombin as a substrate, it has been found that either the platelet or its plasma co-factor may be a limiting factor in the formation of thrombin (in the just named situation). On the basis of these facts, it is not possible to support those theories which regard platelet co-factor from plasma as a complete thromboplastin in itself,[28] or even as a substrate which becomes activated by the platelets.[29] In the preceding experiments, the platelet co-factor from the plasma always acts in conjunction with platelets, never alone, not even after incubation with platelets and calcium. In the experimental work of this thesis, a successful substitution of certain synthetic organic compounds for platelet co-factor of plasma has been achieved. The platelet co-factor which can be obtained from either serum or plasma is apparently similar to or identical to the anti-hemophiliac factor. This view is supported by the observation that partially purified platelet co-factor when added to hemophilic plasma will increase the rate of activation of prothrombin. For some years the defect in hemophiliacs has been considered to be a lack of a plasma component.[30] Tocantins[31,32,33] has stated that the hemophiliac contains or has in his blood large amounts of anti-hemophilic material, but that this is associated with an inhibitor. In studies by Johnson[34] it was also found that an inhibitor can be removed from hemophilic plasma by extracting it with ether and that when this inhibitory extract is returned

to the plasma, the rate of activation of purified prothrombin is decreased. In the work of this thesis, the question of the inhibitor is not brought into focus.

CHAPTER I

METHODS AND MATERIALS

This chapter deals primarily with the technique used in assaying thromboplastic materials. Both the technique and the preparation of materials are described in detail. This is necessary because of the importance of establishing the exact conditions of these experiments as well as the standards of purity for the reagents. Variations in technique yield variations in the purity of products as well as variations in the rate of the reaction of the reagents.

In this laboratory, standard reagents such as prothrombin, fibrinogen, incubation mix and the like were prepared under assignment by various members of the laboratory staff. These were constantly checked against control values so that a "standardized reagent system" was always available.

A. PURIFIED PROTHROMBIN

Prothrombin is a glycoprotein having a molecule weight of about 65,000 which is found associated with the alpha 3 fraction of the plasma. The concentration of prothrombin in whole blood is about 0.007%. It is a protein sensitive to weak acid, weak alkali and most chemical reagents in the laboratory. Thus, its separation from the other plasma proteins represents a special undertaking.[35,36] The use of alcohol, ether or salts has thus far not yielded a fraction containing prothrombin in sufficient purity for use in studying its properties. The method used is that of Seegers and in essence consists of the following principles: Blood is mixed with a special anticoagulant designed to keep the salt concentration of the plasma low. The formed elements are removed by centrifugation. The prothrombin is precipitated isoelectrically from diluted plasma, adsorbed on magnesium hydroxide, liberated from the magnesium hydroxide by decomposing the latter with carbon dioxide, and concentrated by ammonium sulfate and isoelectric fractionation.

Further purification is done by adsorption on barium carbonate. This product is the central reagent, the substrate, in all the assays done.

1. IMPURITIES OF PROTHROMBIN[35]

Probably the most important and significant impurity of the prothrombin preparation is that of Ac-globulin. This may be there in quantities up to 0.5% of the dry weight. Although this cannot be removed by a physical or chemical procedure, it is possible to inactivate the Ac-globulin by heating the prothrombin solution at 53°C entigrade for thirty minutes.

B. PURIFIED THROMBIN

A highly purified thrombin preparation is obtained by the auto catalytic activation of purified prothrombin in 25% sodium citrate.[37] Activation by this means makes it possible to avoid the introduction of calcium,

inadequately purified Ac-globulin and thromboplastin.

C. PURIFIED FIBRINOGEN

It is essential in analytical experiments that there be a constant source of active purified fibrinogen in order to insure accuracy and repeatable results. Commercial preparations are usually not adequate, containing only from 75 to 90% clottable protein or even less. A simple method to obtain fibrinogen is used in this laboratory.[38] It is based upon the observation that fibrinogen is not completely soluble in cold plasma. This method, carefully carried out, regularly yields a product which after freezing and thawing, remains clear for many hours and is relatively free of prothrombin, fibrinolytic enzymes and other elements concerned in the clotting reaction.

D. SILICONING

The method used here was with General Electric Drifilm. Clean glassware without scratches was rinsed in General Electric Drifilm. The glassware which was rinsed was then rinsed with distilled water at least two times. This glassware was then placed in a dry oven and heated at 100°C entigrade for two hours.

E. BOVINE PLATELET EXTRACT

Platelet extract is prepared by the method of Seegers, Fell, Alkjaersig and Johnson.[40] Platelets are obtained by differential centrifugation, washed and fragmented. The extract obtained has minimal thromboplastic activity and serotonin. It contains appreciable quantities of platelet factor 3, a partial thromboplastin which together with the anti-hemophilic globulin of the plasma form a complete thromboplastin.

F. INCUBATION MIXTURE[41,42]

This mixture consists of the following components:

Acacia (15% solution)	2 parts
Imidazole buffer (pH 7.2 - 7.4)	1 part
$CaCl_2$ (0.70%)	2 parts
Na Cl (0.85%)	4 parts

This incubation mixture is used in the dilution of the thrombin derived in the conversion mixture. Its purpose is to stabilize the reaction of the conversion of fibrinogen to fibrin.

1. IMIDAZOLE BUFFER

This is prepared by dissolving 1.75 grams of imidazole in 90 ml. of 3.1 N HCl and then diluted to 100 ml. volume with distilled water. The pH can be adjusted with a few drops of strong HCl or Na OH if it is not 7.2 or 7.4 which is the optional range for the determinations and use of this buffer.

G. THROMBOPLASTIN

The source of thromboplastic material used as control for these experiments is from bovine lung. This is a crude extract of pulmonary tissue but is relatively free from antithrombic material. Phenol is added in the concentration of o.5%. This product usually diluted before adding to an assay system. The ideal dilution is 4 parts physiological saline to 1 part thromboplastin.

H. THE THROMBIN TITER METHOD FOR THE ASSAY OF THROMBOPLASTIN ACTIVITY

A method of assaying thromboplastin activity by measuring thrombin titer was described by Johnston et al.[27] A modification of this procedure is used in this work to measure the relative activities of thromboplastic materials. The principle of this method is to provide all except one of the necessary factors in the activation of the purified substrate. The thromboplastic factor to be assayed then becomes the limiting factor or the variable. The rate and yield of activation are measured by determining thrombin

activity which is measured as a function of its conversion of fibrinogen to fibrin. This procedure will be illustrated here in detail.

The conversion mixtures: A control is set up in which the total amount of prothrombin is measured by a modified two stage procedure.[43] One ml. of a purified substrate, prothrombin, usually containing about 3000 units per ml. is placed in a silicone treated tube. The other factors are then added: calcium chloride 0.5 ml., .163M, a saline blank 0.85%, 0.5 ml., and finally the lung thromboplastin 0.5 ml. A stop watch is started at this time and the conversion mixture is incubated at 28°Centigrade.

Example of control conversion mixture:

Prothrombin 3000 units/ml.		1.0 ml.
Platelet factor		0.5 ml.
Calcium chloride 0.163M		0.5 ml.
Saline 0.85%		0.5 ml.
Lung thromboplastin		0.5 ml.
Conversion Mix I	Total	3.0 ml.

Inasmuch as partially purified platelet factor has some minimal thromboplastic activity, a second control is usually performed to test for this activity. A conversion mixture is prepared as in the first control omitting lung thromboplastin and substituting 0.5 ml. of 0.85% saline for it in the mixture. A stop watch is started at the exact moment the mixture is made. Example of conversion mixture to test for platelet thromboplastin activity:

Prothrombin 3000 units/ml.		1.0 ml.
Platelet factor		0.5 ml.
Calcium Chloride 0.163M		0.5 ml.
Saline 0.85%		1.0 ml.
Conversion Mix II	Total	3.0 ml.

The experimental assays are done in a similar manner. However, lung thromboplastin is excluded. A sample conversion mixture to test a compound for thromboplastin activity is given:

Prothrombin 3000 units/ml.		1.0 ml.
Platelet factor		0.5 ml.
Calcium chloride 0.163 M		0.5 ml.
Saline 0.85%		0.5 ml.
Organic compound X mg./ml.		0.5 ml.
Conversion Mix III	Total	3.0 ml.

As in the controls a stop watch is started at the moment the conversion mixture is prepared.

1. CLOTTING OF FIBRINOGEN TO MEASURE THE THROMBIN CONCENTRATION

At measured intervals, usually two minutes apart if activation is rapid or longer intervals if activation is slow,* 0.3 ml. of the diluted conversion mixture is removed and added to 0.075 ml. of fibrinogen, previously pipetted into small glass tubes of dimensions 12 by 75 mm. The clotting end point, the same as that of the two stage test for prothrombin, is determined accurately by means of a second stop watch started at the instant of mixing of the fibrinogen and the conversion mixture. The end point is observed as the first formation of definite granules in the test solution. Invariably, a cloudiness develops in the previously clear solution as the end point is approached. This is followed very shortly by the granularity. In the case of a true end point, the granules are followed by the formation of fibrin strands and a definite clot formation in a few seconds; whereas, in the case of a false end point, due to variations in the acacia, the granulation is not followed by the formation of a clot.

*A 0.1 ml. aliquot of the conversion mixture is removed and diluted to the desired dilution. The first dilution is made in 0.85% saline.

The second dilution is made in the incubation mixture (previously described). For example if a dilution of 1000 is desired, 0.1 ml. of the conversion mixture is diluted in 3.9 ml. of saline. This gives a dilution of 40. A 0.1 ml. of this mixture is then pipetted into 1.9 ml. of incubation mixture. This gives a dilution of 800.

2. CALCULATIONS

The number of dilutions of the prothrombin is first determined.

1. Diluted 3 times with the conversion mixture.
2. Diluted X times with saline.
3. Diluted Y times with incubation mixture.
4. Diluted 1.25 times with fibrinogen.

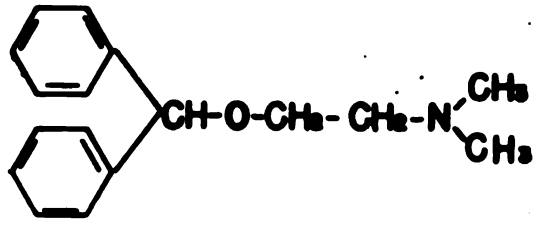
The total dilution equals: 3 x X x Y x 1.25. The total dilution is multiplied by the unit conversion factor which depends upon the clotting time. This is determined by the use of a standard table.[44] If the clotting time were 13 seconds, the factor would be 1.2. The total number of thrombin units would then equal 1.2 (unit conversion factor) times the total dilution.

The unit of standard thrombin is defined as that amount which will clot 1 ml. of standardized fibrinogen in 15 seconds under standard conditions.

The rate of activation of prothrombin is evaluated by plotting a curve of thrombin yield (ordinate) against time abscissa. The yield is determined by extrapolating from the assymptote to the ordinate. This is illustrated in figures 1-10.

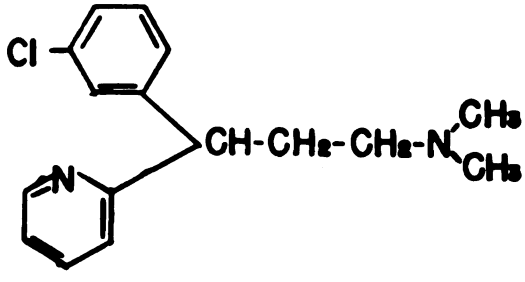
I. The following organic compounds were tested for their thromboplastic activity:

1. BENADRYL



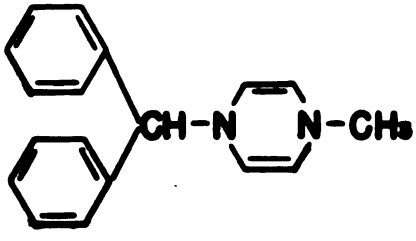
2(Benzhydryloxy)-N,N-dimethylethylamine

2. CHLORTRIMETON



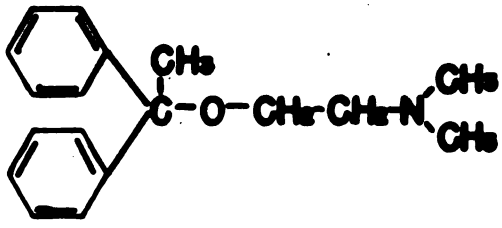
1(p-Chlorophenyl)-1-(2-pyridyl)-3-dimethyl amine propane

3. CHLORICYCLIZINE



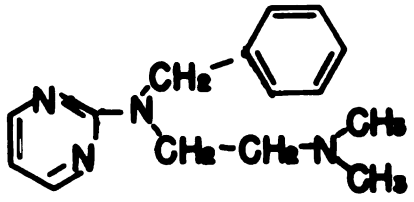
1-(4-Chlorobenzhydryl)-4-methylpiperazine

4. DECAPRYN



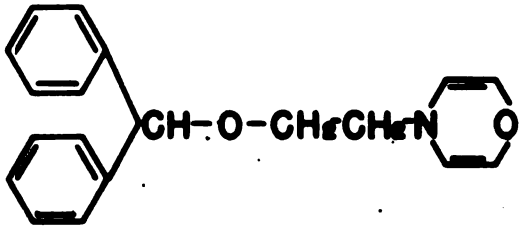
2-(α(2-Dimethylaminoethoxy)-α-methyl benzyl)pyridine

5. METRAMINE



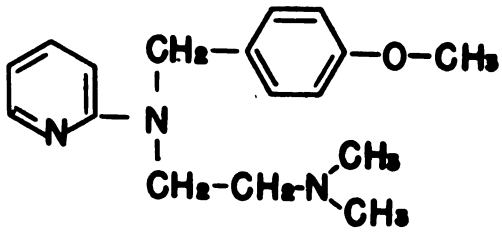
2(Benzyl(2-dimethylaminoethyl)amino)pyrimidine

6. LINADRYL



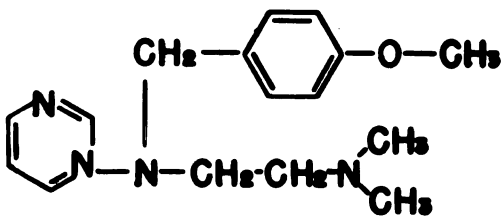
4-(2-(Benzhydryloxy)ethyl)morpholine

7. NEOANTERGAN



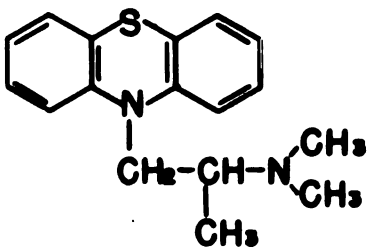
2-((2-Dimethylaminoethyl)(p-methoxybenzyl) amino)pyridine

8. NEOHETRAMINE



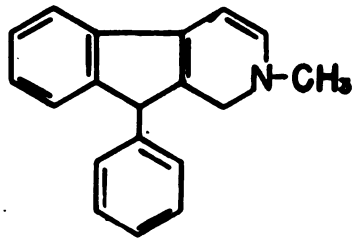
2-((2-Dimethylaminoethyl)(p-methoxybenzyl) amino)pyrimidine

9. PHENERGAN



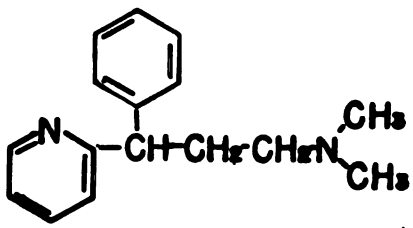
10-(2-Dimethylamine-1-propyl)phenothiazine

10. THEPHORIN (PHENINDAMINE)



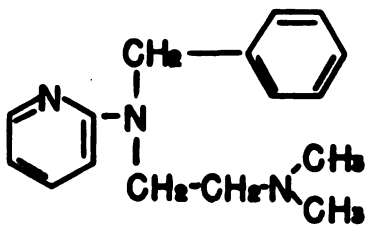
2-Methyl-9-phenyl-2-3-4-9-tetrahydro 1-pyridindene

11. TRIMETON



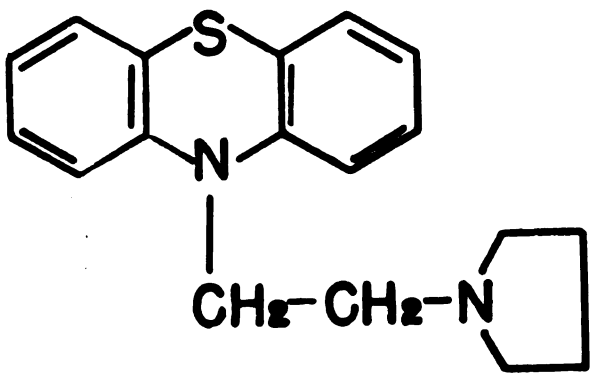
2-(α(2-Dimethylaminoethyl)benzyl) pyridine

12. PYRIBENZAMINE



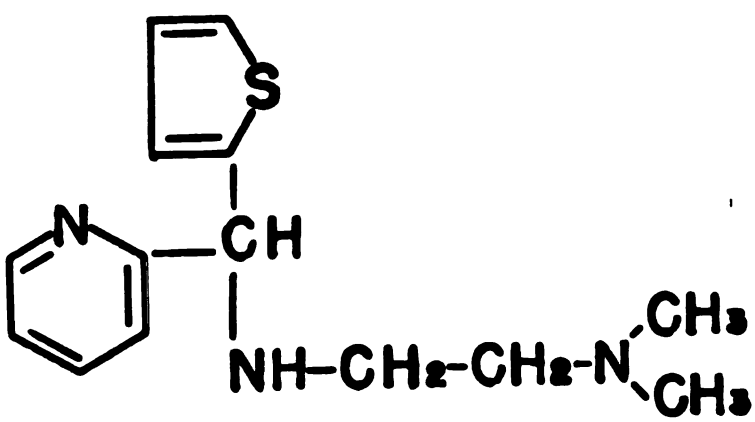
2-(Benzyl(2-dimethylaminoethyl)amino)pyridine

13. PYRROLAZOTE



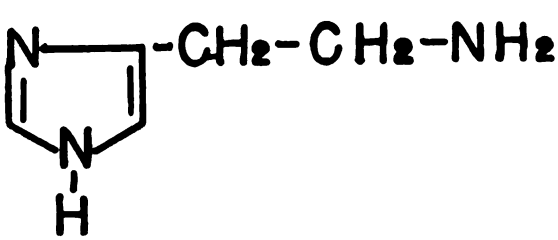
10(2-(1-Pyrrolidyl)ethyl)phenothiazine

14. THENYLENE



2-((2-Dimethylaminoethyl)-2-thenylamino pyridine

15. HISTAMINE



CHAPTER II

A. HISTAMINE, A THROMBOPLASTIC AGENT

The initial pilot experiments which demonstrated that histamine might actually have thromboplastic activity, were performed by substituting histamine for lung thromboplastin in the previously developed system for thromboplastic assay, vide supra. A series of tubes was set up in which the activation of a standard prothrombin product was followed during activation with lung extract thromboplastin. The standard incubation mixture was as follows:

Prothrombin (3750 units)	1.0 ml.
Platelet factor	0.5 ml.
Calcium chloride .15M	0.5 ml.
Saline .86%	0.5 ml.
Lung Thromboplastin (TPLN)	0.5 ml.

Conversion Mixture I

A second mixture was set up substituting histamine for lung thromboplastin:

Prothrombin (3750 units)		1.0 ml.
Platelet factor		0.5 ml.
Calcium chloride		0.5 ml.
Saline 0.85%	base	0.5 ml.
Histamine diphosphate .002 mg./ml.		0.5 ml.

Conversion Mixture III

The result of this type of experiment is shown in a summary graph in Figure I. From this it can be seen that histamine apparently has the ability to activate variable amounts of prothrombin after short periods of time.

FIGURE I

HISTAMINE, AN ACTIVATOR OF PROTHROMBIN

Curves showing the conversion of prothrombin to thrombin with various concentrations of Histamine, against time.

Prothrombin (3750 units)	1.0 ml.
Platelet extract	0.5 ml.
Saline	0.5 ml.
Calcium Chloride .163M	0.5 ml.
Histamine X mg. (base)/ml.	0.5 ml.
Conversion Mix III	Total 3.0 ml.

Curve A: Thromboplastin
Conversion Mix I

Curve B: Histamine 0.002 mg. (base)/ml.
Conversion Mix III

Curve C: Histamine 0.01 mg. (base)/ml.
Conversion Mix III

Curve D: Histamine 0.1 mg. (base)/ml.
Conversion Mix III

Curve E: Platelet extract
Conversion Mix II

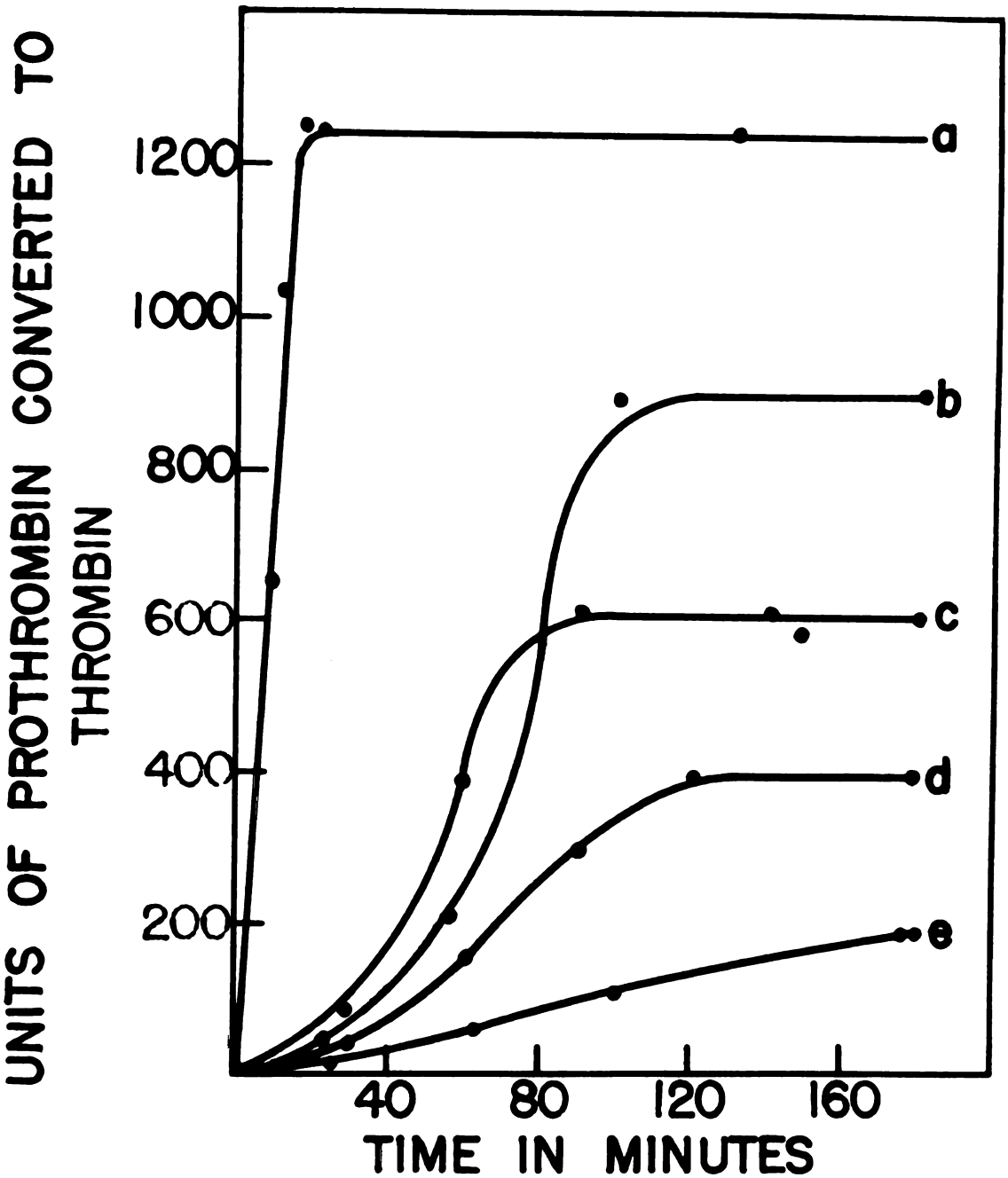


FIG. 1 HISTAMINE, AN ACTIVATOR OF PROTHROMBIN

It was soon found that various concentrations of histamine gave different activation curves. It was also apparent from one assay to another that activation with histamine was erratic and was difficult to repeat quantitatively. Under the conditions of this assay high concentrations of histamine gave poorer yields than low concentrations. The optimum concentration was 0.002 mg. (base)/ml. of histamine di-phosphate. The factors which lead to these irregularities must be manifold; however, many were eliminated in subsequent experiments.

It was observed that adding calcium chloride to a buffer solution (imidazole buffer a pH 7.2 to 7.4, see section on methods and materials) gave results which were more constant. It was also decided that the total volume of histamine should be limited to 0.5 ml. It was also found that histamine must be freshly prepared in order to be active. This was an empirical observation and detailed stability studies were not done, although it was shown that in 72 hours approximately two-thirds of the activity of the histamine was gone. Section C of this chapter gives additional data.

B. QUANTITATIVE RELATIONSHIPS BETWEEN HISTAMINE AND CALCIUM CHLORIDE

The optimum concentration for histamine and calcium chloride were determined by empirically selecting a series of tubes with graded concentrations of calcium chloride and histamine and determining the total amount of prothrombin converted to thrombin in a 60 minute period. Group I contained 0.002 mg. histamine base per ml., Group II contained 0.01 mg. base per ml., Group III contained 0.1 mg. base per ml., and Group IV contained 1.0 mg. base per ml. To the four tubes in each group were added 0.001 M, 0.01 M, 0.02 M, and 0.1 M calcium chloride, respectively. The results are plotted in Figure 2. From these data it was decided that

the optimum concentration of histamine is 0.002 mg. (base)/ml. and that the optimum concentration of calcium chloride is in the neighborhood of .10 M. It was therefore decided to use the calcium concentration standard in this laboratory, which is 0.163 M.

It can be seen that extreme concentrations of calcium chloride did not give high yields of thrombin at any histamine concentration used. Each group of assays gave a well shaped curve. Histamine concentrations, however, showed maximum activation at the extremes of the given concentrations: 0.002 mg./ml. and 1.0 mg./ml.

The significance of these variations in activation yield as a function of the concentrations of calcium chloride and histamine escapes this author. At the present time these data can only be considered as empirical observations and utilized as such. Additional data might well reveal significant aspects of the activation mechanism.

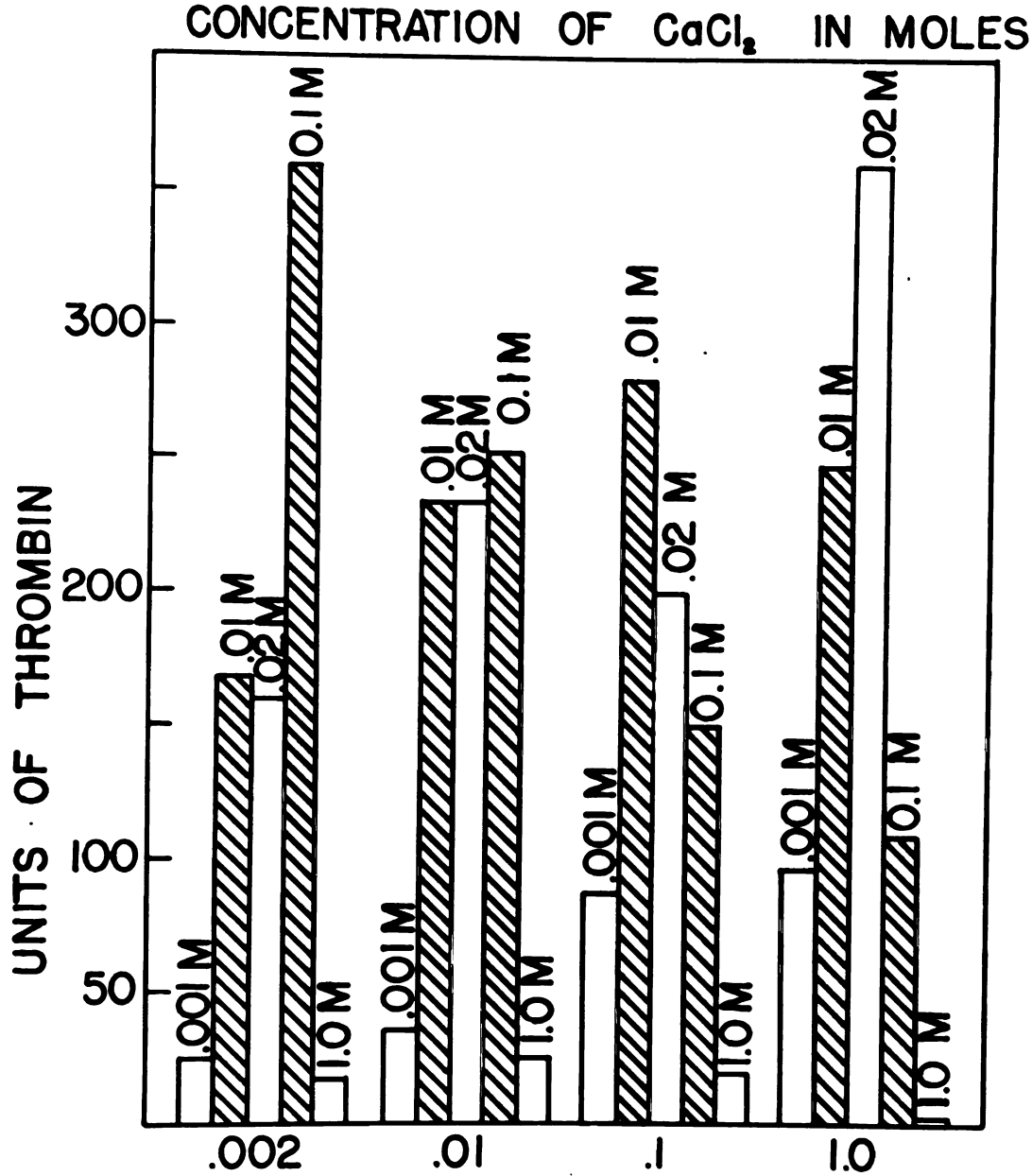
FIGURE 2

VARIATIONS IN CONCENTRATION OF CALCIUM CHLORIDE AND HISTAMINE

A multiple graph showing variations in thrombin yield with respect to various concentrations of calcium chloride and histamine (base).

Prothrombin	1.0 ml.
Platelet extract in distilled H_2O	0.5 ml.
Calcium Chloride XM	0.5 ml.
Saline 0.85%	0.5 ml.
Histamine Y mg. (base)/ml.	0.5 ml.

Incubation time: 60 minutes.



VARIATIONS IN CONCENTRATION OF CALCIUM CHLORIDE AND HISTAMINE BASE

FIG. 2

C. THE STABILITY OF SALINE DISSOLVED HISTAMINE PREPARATIONS

The question of the erratic behaviour of histamine with respect to its ability to activate prothrombin, plagued us for a number of months. Although the addition of histamine to a control assay always showed an increase in yield, the amount varied from a few percent, where the statistical significance might be in question, to the maximum yield. At that time it was decided to do a preliminary stability study of histamine in solution. One sample of histamine solution, prepared as in the section on methods and materials was aged for 27 days; another sample was made up fresh. A series of assays was run with two controls, lung thromboplastin for maximal yield, and platelet extract for minimum yield.

The results of these experiments showed that the activity of histamine with respect to activation of prothrombin decreased as the solution aged. (See Figure 3.).

Experiments seeking the nature of the change in histamine solutions were not pursued. Although it would be interesting to note whether the histamine had lost its classical pharmacological properties and whether the inactivation of the histamine occurred as a result of a purely physical effect, i.e. light, or a chemical effect mediated by the saline or hydronium ions. It may be noted that histamine solutions ordinarily maintain their usual physiological activities in solution for considerable periods of time. The exact nature and mechanisms of the change in the histamine were not considered vital to the objective pursuit of these experiments.

FIGURE 3

THE DETERIORATION OF HISTAMINE SOLUTION WITH RESPECT TO THROMBOPLASTIN ACTIVITY

Histamine .002 mg. (base)/ml.

Prothrombin 4500 units/ml.

Curve A: Lung thromboplastin control.
(Conversion mix I in methods and materials H.)

Curve B: Freshly prepared histamine solution (in normal saline).
(Conversion mix III in methods and materials H.)

Curve C: Histamine solution, 27 days old.
(Conversion mix III in methods and materials).

Curve D: Platelet extract control.
(Conversion mix II in methods and materials H.)

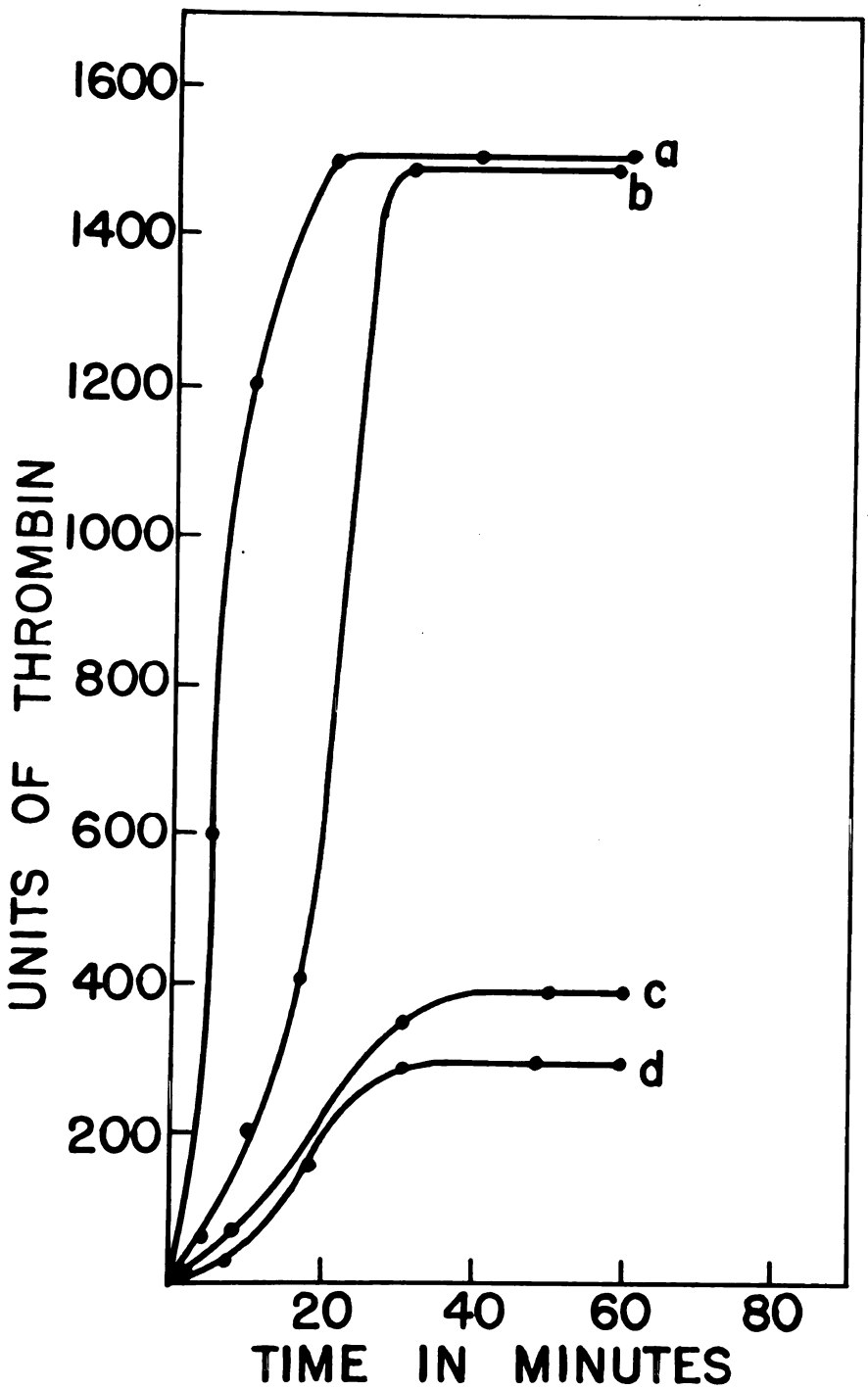


FIG. 3 THE DETERIORATION OF HISTAMINE SOLUTION WITH RESPECT TO THROMBOPLASTIN ACTIVITY

D. THE RELATIONSHIP TO PLATELET EXTRACT (PLATELET FACTOR 3)

Further analyses of this system were carried out, and the action and contribution of the individual components of the system were tested in detail by the method of elimination experiments. Calcium chloride solutions in the concentrations used in this assay showed no observable thromboplastic activity. Platelet extract in the presence of 0.163 M calcium chloride showed a minimum slow type activation of the prothrombin. This phenomenon has been noted previously by other workers. It is to be **stressed that the maximum quantity of prothrombin** activated here is in the order of magnitude of 33% and usually 15 - 20%.

Aqueous solutions of histamine alone did not possess the power to convert prothrombin to thrombin, nor did saline solutions of the same. The addition of the calcium chloride component did not yield any significant thromboplastic activity. No thromboplastic activity was demonstrated until platelet extract was added to the assay. It must then be concluded that histamine acts in conjunction with platelet extract in some manner so as to activate prothrombin. A summary of these experiments is shown in Figure 4.

FIGURE 4

THE EFFECT OF PLATELET AC-GLOBULIN AND THE ACTIVATION OF PROTHROMBIN

Prothrombin 3600 units/ml.

Histamine 0.002 mg. (base)/ml.

Curve A: Thromboplastin control.
(Conversion mix I in Methods and Materials H).

Curve B: Histamine plus calcium chloride and platelet extract.
(Conversion mix III in Methods and Materials H).

Curve C: Platelet extract control (minus histamine or lung thromboplastin). (Conversion mix II in Methods and Materials H).

Curve D: Histamine plus calcium chloride (no lung thromboplastin or platelet extract). (Conversion mix III in Methods and Materials H. Saline blank substituted for platelet extract).

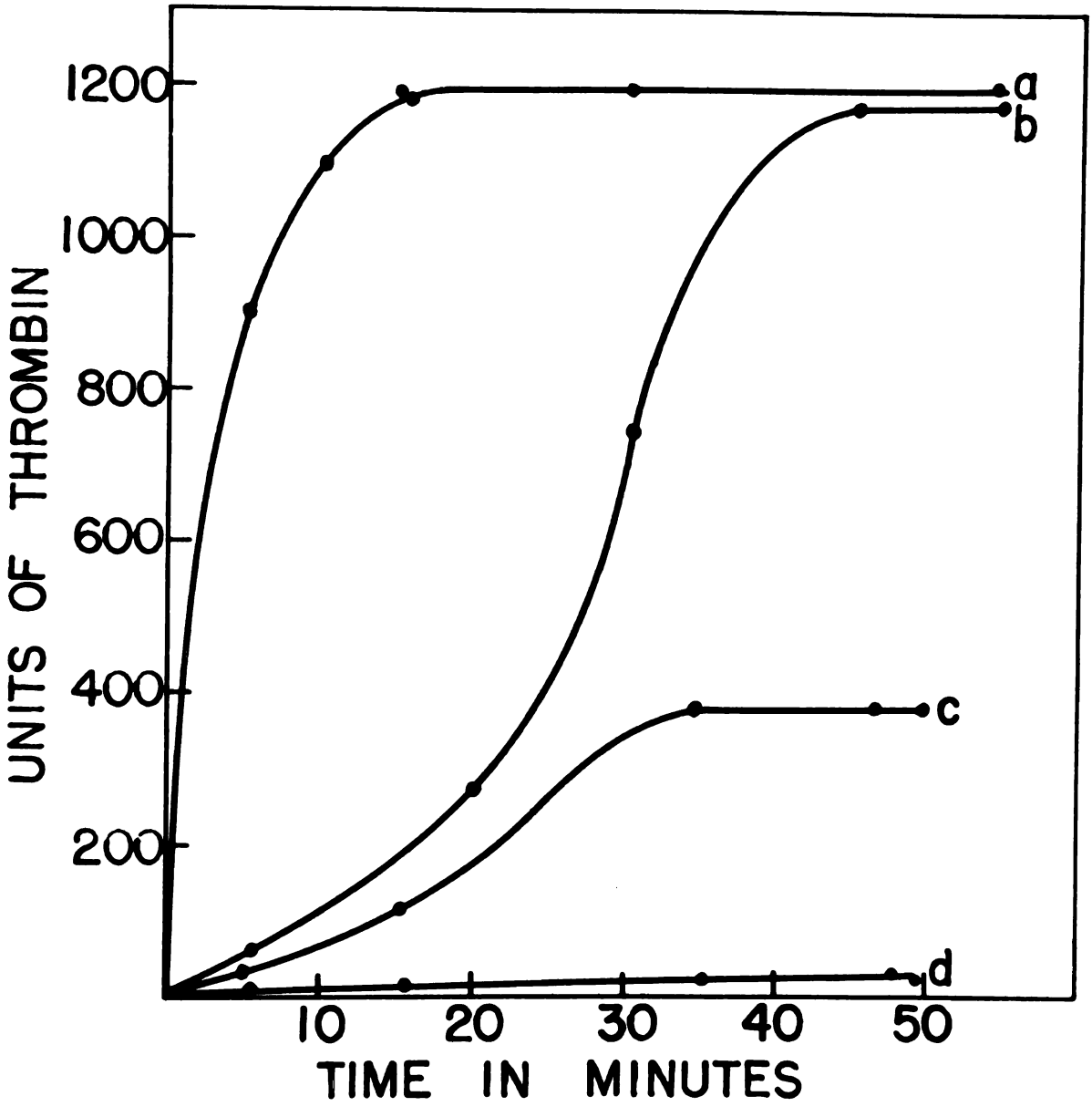


FIG. 4 THE EFFECT OF PLATELET AC-GLOBULIN AND THE ACTIVATION OF PROTHROMBIN

E. THE RELATIONSHIP OF HISTAMINE TO PLASMA AC-GLOBULIN

From the results of the purification work of prothrombin, it is known that thrombin activity arises from prothrombin. The final proof of this being in the autocatalytic activation of purified prothrombin in 25% sodium citrate solution. However, purified prothrombin (as ordinarily used in these experiments) does contain an impurity which is most certainly plasma Ac-globulin. This is present in maximum quantities of 0.5%.

It was necessary in this series of experiments primarily to establish the relationship of histamine to this impurity, and secondly to Ac-globulin in general. Two experiments were then performed.

1. If purified prothrombin is heated at 53°Centigrade for thirty minutes, no further Ac-globulin activity can be demonstrated. A lot of purified prothrombin was so prepared. The results of the activation of Ac-globulin free prothrombin are demonstrated in Figure 5. The activation by lung thromboplastin compares with the activation of ordinary purified prothrombin. However, platelet extract shows no appreciable thromboplastic activity after ninety minutes of incubation. Histamine shows only less than 30% activation after sixty minutes of incubation.

From this we see that the prothrombin impurity, namely plasma Ac-globulin, has an important relationship to the organic compound or plasma platelet co-factor and platelet extract. The nature of this relationship will be discussed further. On the other hand, characteristics of activation by lung thromboplastin vary little if any from activation by lung thromboplastin of a substrate containing Ac-globulin.

2. To further test the relationship of histamine with plasma Ac-globulin, a lot of prothrombin containing relatively large amounts of Ac-globulin was made by the method of Seegers, Loomis and Vandenbelt.[45]

This material was used as a substrate in a series of assays with histamine and lung thromboplastin. The results of these experiments are shown in Figure 6.

It can be seen here that activation by lung thromboplastin was not appreciably changed from other substrates containing (a) no Ac-globulin, (b) less than 0.2% Ac-globulin. Activation by histamine of this substrate (Ac-globulin rich prothrombin) is similar to histamine activation and purified prothrombin containing trace amounts of Ac-globulin.

We may then conclude that:

1. Plasma Ac-globulin is necessary for the rapid activation of prothrombin by the histamine - platelet extract complex.

2. Ac-globulin is utilized in a manner reminiscent of data with plasma co-factor (AHG).[29]

In summary, the following principles have been established:

1. Histamine, an organic amine, acts like plasma platelet co-factor (co-factor I) and has a thromboplastic nature. Thus, histamine can mediate the conversion of prothrombin to thrombin.

2. Histamine cannot act alone in the conversion of prothrombin to thrombin, but requires a platelet factor in order to act. (See Figure 4).

3. The histamine platelet complex requires yet another factor for its effective (rapid) conversion of prothrombin, namely, plasma Ac-globulin.

(a) The quantity of plasma Ac-globulin necessary for this reaction is less than 0.5% of the total prothrombin.

FIGURE 5

THE EFFECT OF PLASMA AC-GLOBULIN ON THE ACTIVATION OF PROTHROMBIN*

Prothrombin 3900 units/ml.

Histamine 0.002 mg. base/ml.

Curve A: Thromboplastin control with heated prothrombin substrate. Conversion Mix I

Curve B: Histamine plus heated prothrombin substrate. Conversion Mix III

Curve C: Platelet extract control plus heated prothrombin. Conversion Mix II

*Prothrombin heated at 53° Centigrade for thirty minutes contains no Ac-globulin activity.[37]

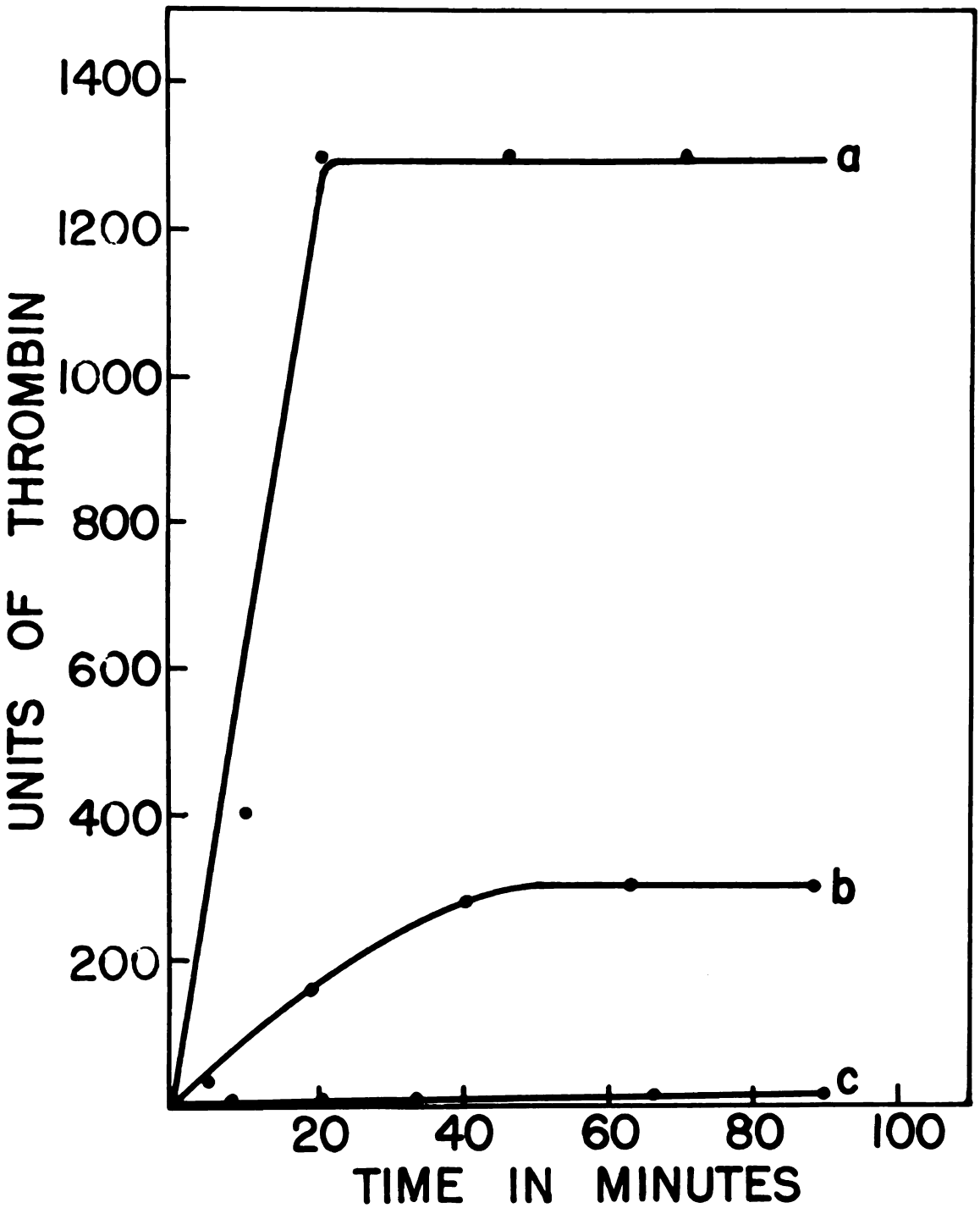


FIG. 5 THE EFFECT OF PLASMA AC-GLOBULIN ON THE ACTIVATION OF PROTHROMBIN

FIGURE 6

THE EFFECT OF A PROTHROMBIN SUBSTRATE CONTAINING HIGH QUANTITIES OF PLASMA AC-GLOBULIN

Prothrombin is eluted from Mg $(OH)_3$ without $(NH_4)_2SO_4$ fractionation. It is all precipitated at an $(NH_4)_2SO_4$ concentration of 60%. The result is a prothrombin product containing high quantities of plasma Ac-globulin.[45]

Prothrombin 4200 units/ml.

Histamine 0.002 mg. (base)/ml.

Curve A: Thromboplastin control plus Ac-globulin rich prothrombin substrate. Conversion Mix I.

Curve B: Histamine plus Ac-globulin rich prothrombin substrate. Conversion Mix III.

Curve C: Platelet extract control plus Ac-globulin rich prothrombin substrate. Conversion Mix II.

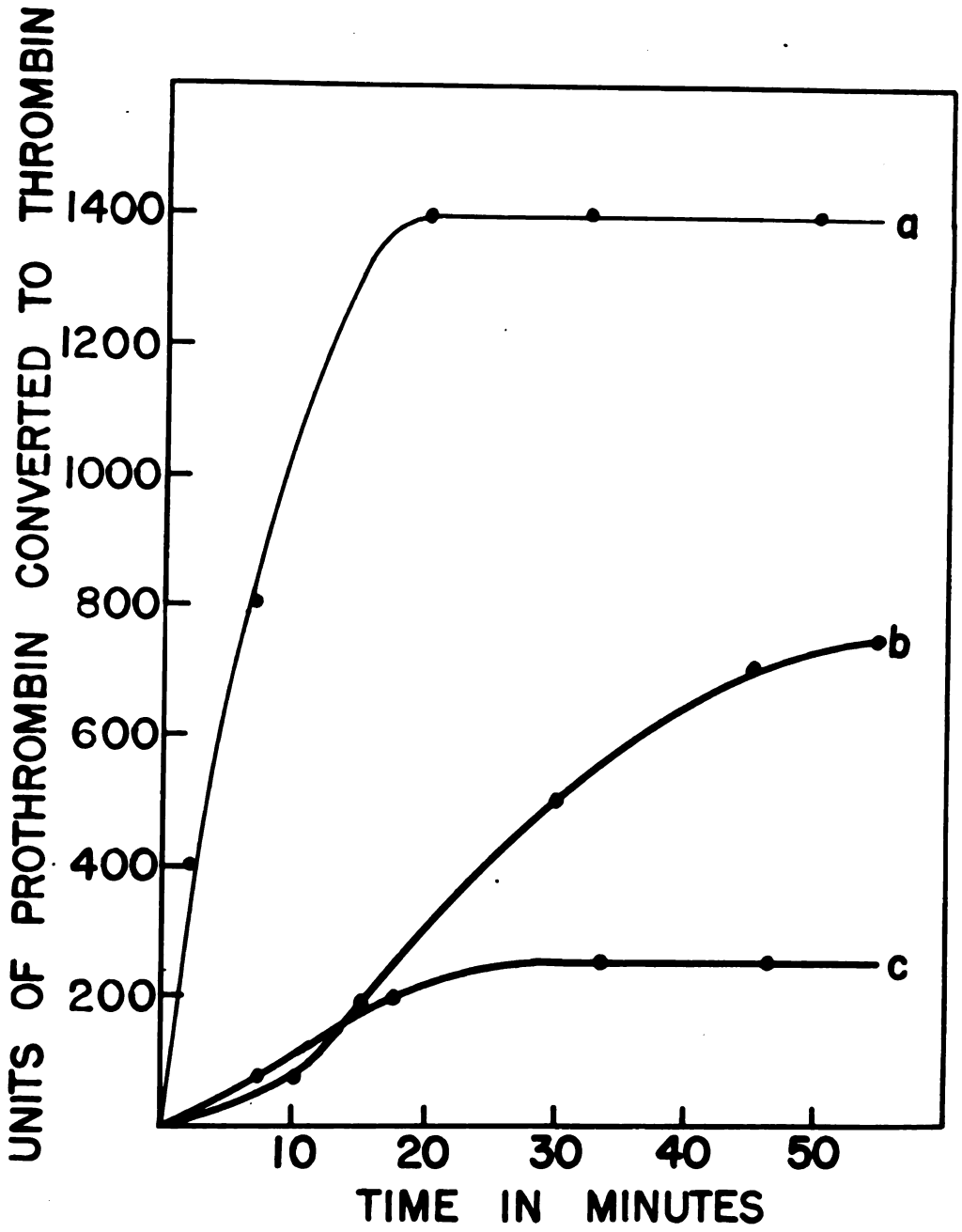


FIG. 6 THE EFFECT OF A PROTHROMBIN SUBSTRATE CONTAINING HIGH QUANTITIES OF PLASMA AC-GLOBULIN

CHAPTER III

A. SURVEY EXPERIMENT OF THE ANTIHISTAMINES

Although histamine has previously been implicated in the clotting mechanism, other investigators have supposed that it acts in relation to platelet disintegration,[21] to adrenochrome[15] or to heparin.[19] The contention here is that histamine has direct thromboplastic activity by virtue of its chemical structure (possibly apart from that giving rise to its classical physiological properties). On this basis, it might be supposed that the homologues and analogues of histamine, namely the antihistamines, might also have thromboplastic properties.

A survey of some fourteen commercial antihistaminic compounds (generally supplied by their manufacturers) was conducted in which these compounds were assayed by the method given for thromboplastic activity. One modification of the technique was made. All of the compounds were incubated semi-simultaneously and the amounts of thrombin yield were determined at sixty minutes of incubation.

One tube was set up as a control containing only platelet extract. A second tube contained histamine as in the previous experiments. Thirdly, an evaluation of the 1 ml. prothrombin dilution was made with lung thromboplastin. The following antihistamines were assayed:

1. Benadryl:(diphenbydramine H Cl) or 2 (Benzhydryloxy) N, N - dimethyl ethyl amine - H Cl.

2. Chlortrimeton:1 (p - chlorophenyl) - 1 - (2 - pyribyl) - 3 - dimethyl amino propane maleate.

3. Chlorcyclizine:(Perazil) 1 - (4 - Chlorobenzhydryl) - 4 - methylpiperazine di H Cl.

4. Decapryn:succinate 2 - (L-- 2 - dimemethyl-aminoethoxy - L - methylbenzyl) pyridine succinate.

5. Hetramine:- 2 - Benzyl (2 - dimethylaminoethyl) amino pyrimidine H Cl.

6. Linadryl:4 - 2 - (Benzhydryloxyethyl morpholine H Cl.

7. Neoantergan:2 - (2 - Dimethylaminoethyl) (p-methoxybenzyl) - amino pyridine.

8. Neohetramine:2 - (2 - Dimethylaminoethyl) (p - methoxybenzyl) amino pyrimidine H Cl.

9. Phenergan:10 - (2 - Dimethylamino - 1 - propyl) phenothiazine H Cl.

10. Trimeton:(prophenpyrdamine 2 - D (2 - dimethylaminoethyl) benzyl pyridine.

11. Pyribenzamine:2 - Benzyl (2 - dimethylaminoethyl) amino pyridine H Cl.

12. Pyrrolazote:10 - 2 - (1 - Pyrrolidyl) ethyl phenothiazine H Cl.

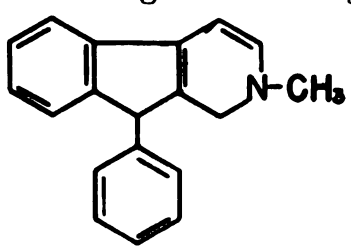
13. Thenylene:2 - 2 - Dimethylaminoethyl) - 2 - thenylamino - Pyridine.

14. Thephorin,Phenindamine:2 - Methyl - 9 - phenyl - 2, 3, 4, 9 - turahydro - 1 - pyridindene.

The total amount of thrombin yield for each compound was then computed. The results were expressed in the following ways: the ratio of the yield per compound to that of the platelet control, the percentage of the original prothrombin converted to thrombin, and the additional amount of prothrombin converted due to the action of the organic compound with the platelet extract. The platelet extract synergism was derived by subtracting the percentage of the thrombin produced by the platelet extract control from the amount produced by the compound plus platelet extract. The results are found in Table I.

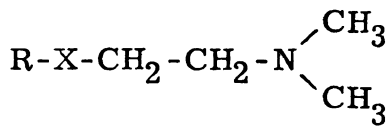
The findings are immediately apparent. Five compounds showed thromboplastic activity in magnitudes which must be statistically significant. These are histamine, benadryl, decapryn, linadryl and thephorin.

The structural formulae, as well as the chemical descriptions of all of the above mentioned compounds are shown in the section on methods and materials. A brief analysis of these formulae reveals that the active histamine analogues occur in two general categories, the pyridindene derivatives:



2-Methyl-9-phenyl-2-3-4-9-tetrahydro 1-pyridindene

and the dimethyl amino ethyl - X compounds:



Dimethyl amino ethyl-X

The latter are divided in three categories, where the X, or bridging element (between the dimethyl amino ethyl and the cyclical portion of the molecule), may be substituted by any one of three elements, namely: carbon, nitrogen or oxygen. From the survey presented, we see that one pyridindene derivative and only the dimethyl amino ethoxy compounds have thromboplastic activity. The significance of these chemical structures will be discussed further. The portion of the antihistamine molecule having thromboplastic activity has then been identified.

Surveying the five active compounds (including Histamine) one can see that the most active is Thephorin (phenindamine), a pyridindene compound, followed by Benadryl, Decapryn, Histamine, and Linadryl. It would seem that they activate prothrombin in approximately the same order of magnitude.

TABLE I

A SURVEY OF VARIOUS ORGANIC COMPOUNDS FOR THROMBOPLASTIC ACTIVITY

Column 1. Compound.

Column 2. Actual thrombin yield.

Column 3. Ratio of activity of compound plus platelet extract to that of platelet extract.

Column 4. Percentage of a thrombin yield to that of thromboplastin control.

Column 5. Percentage of thrombin yield minus platelet extract activity.

Compound	Units of Thrombin	Thrombin yield Platelet Activity	% Thrombin	% Thrombin -% Thrombin by Platelet
Thromboplastin	1125			
1. Platelet Factor	240	1.000	21.2	00.0
2. Histamine 0.002 mg base/ml.	900	3.58	76.0	55.0
3. Benadryl 0.004 mg/ml.	1020	4.25	90.5	69.5
4. Chlorcyclizine 1 mg/ml	420	1.75	37.0	16.0
5. Chlortrimeton 1 mg/ml.	438	1.77	37.6	16.4
6. Decapryn 1 mg/ml.	990	4.13	87.5	66.3
7. Hetramine 1 mg/ml.	405	1.68	36.8	15.6
8. Linadryl 1 mg/ml.	800	3.33	70.9	49.7
9. Neo Antergan 1 mg/ml.	475	1.98	42.0	20.8
10. Neo Hetramine lmg/ml.	258	1.08	22.9	01.7
11. Phenergan 1 mg/ml.	3.22	1.34	28.5	07.3
12. Thephorin 1 mg/ml.	1210	5.05	107.0	85.8
13. Prophenpyrdamine 1 mg/ml.	320	1.33	28.4	07.2
14. Pyribenzamine 1 mg/ml.	308	1.28	27.2	06.0
15. Pyrrolazote 1 mg/ml.	300	1.25	26.5	05.3
16. Thenylene 1 mg/ml.	425	1.77	37.6	16.4

FIGURE 7

ACTIVATION CURVES OF FOUR ANALOGUES OF HISTAMINE

Prothrombin 4200 units/ml.

Organic Compounds 1 mg./ml.

Curve T: Thromboplastin control.
Conversion Mix I.

Curve A: Decapryn.
Conversion Mix III.

Curve B: Benadryl.
Conversion Mix III.

Curve C: Thephorin.
Conversion Mix III.

Curve D: Linadryl.
Conversion Mix III.

Curve E: Platelet extract.
Conversion Mix II.

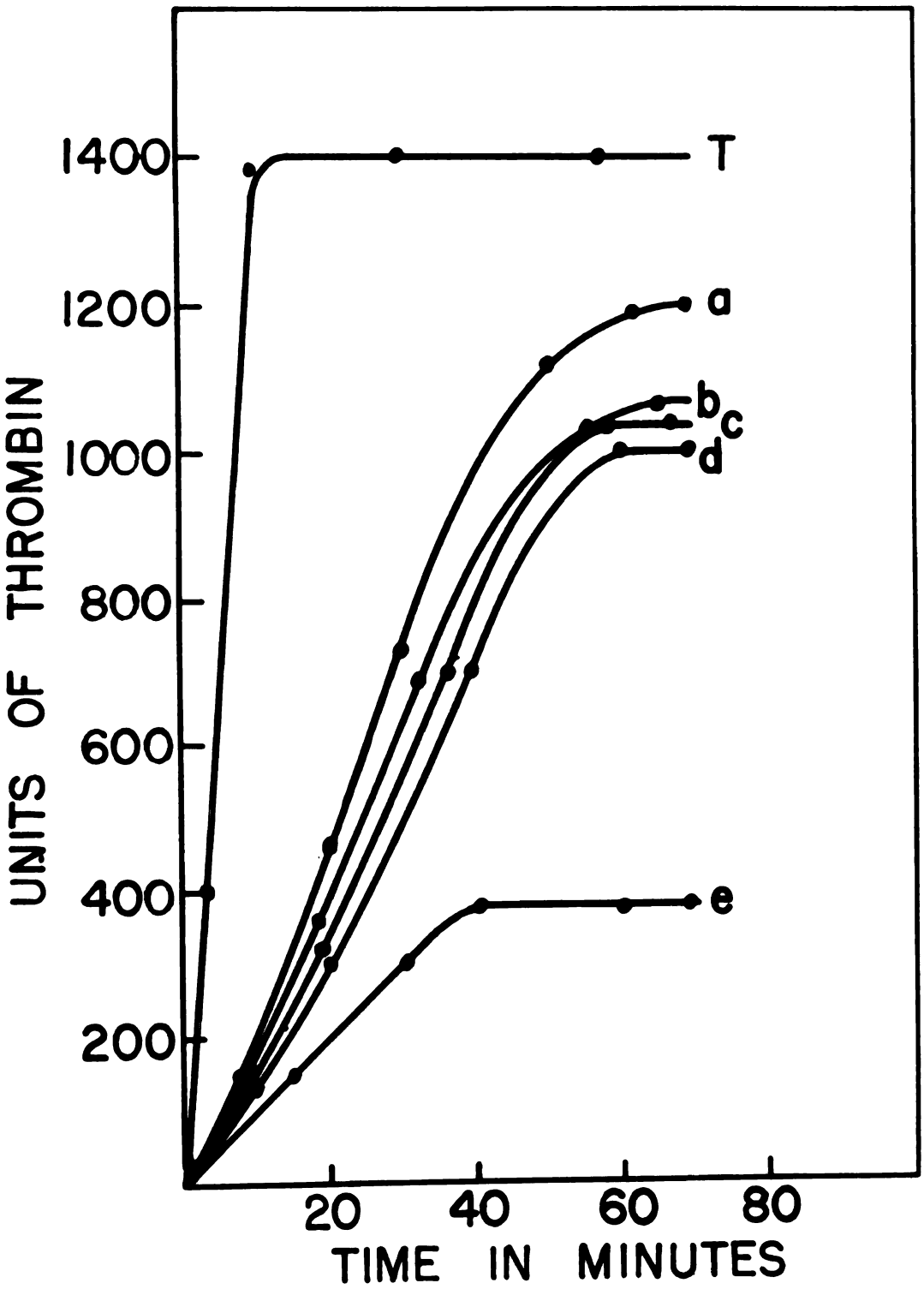


FIG. 7 ACTIVATION CURVES OF FOUR ANALOGUES OF HISTAMINE

B. THROMBIN SEEDING EXPERIMENTS

The question of thrombin yield, activation time and the shape of the activation curve remained a problem to be solved. In spite of the stability studies done with histamine solutions, variations in thrombin yield were obtained which remained beyond deviation of experimental technique. In view of the fact that reagents and techniques were rigidly controlled, it appears reasonable to ascribe these variations to the reactions themselves.

Whereas activation curves with lung thromboplastin were hyperbolic, activation curves obtained with the organic activators were constantly sigmoidal in character. This type activation curve has been noted by other investigators and is the basis of the thromboplastin generation curve by Biggs and Douglas.[46]

Inasmuch as thrombin accelerates the autocatalytic activation of prothrombin in 25% sodium citrate solution, although not in the same order of magnitude with respect to time, the incubated mixture was seeded with purified thrombin. The first pilot experiment contained 200 units of purified thrombin per ml. of stock solution. Subsequently smaller amounts were used. In Figure 8, a series of curves is shown where the thrombin addition is 8 units/ml.

It can be seen here that activation is rapid (at a rate about half to that of lung thromboplastin). The lag phase is reduced, if not eliminated and the curve begins to appear hyperbolic. Activation is complete and the end point coincides with the control (lung thromboplastin).

In order to determine whether thrombin and an organic activator in standard buffered medium containing the standard salts were sufficient to activate prothrombin, a series of experiments were performed to test

this hypothesis. It can be stated that both platelet factor, Ac-globulin and an organic activator must be present in order that thrombin accelerate the activation of the substrate.

If one would speculate as to the action of thrombin under the previously stated conditions, one might think that the action is similar to that occurring in the autocatalysis of prothrombin. However, inasmuch as the time required for autocatalysis is so much greater than that for these rapid activations, it is reasonable to assume that this mechanism alone does not play a significant role here. One might then propose that thrombin accelerates the conversion of plasma Ac-globulin to serum Ac-globulin, and that it is this component which effectively determines the rate of activation of the prothrombin.

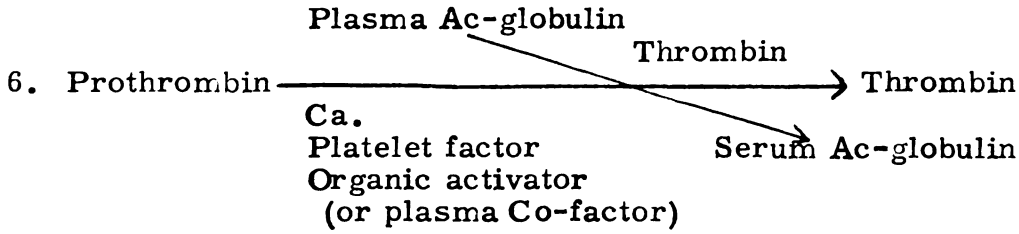


FIGURE 8

THE EFFECT OF THROMBIN SEEDING ON THROMBIN YIELD

Prothrombin 3300 units/ml.

Linadryl 1 mg./ml.

Curve T: Thromboplastin control.
Conversion Mix I.

Curve A: Histamine 0.002 mg. (base)/ml. plus 8 units of thrombin. Conversion Mix III.

Curve B: Linadryl 1 mg./ml plus 8 units of thrombin/ml.
Conversion Mix III.

Curve C: Histamine 0.002 mg. (base)/ml.
Conversion Mix III.

Curve D: Linadryl .1 mg./ml.
Conversion Mix II.

Curve E: Platelet extract control.
Conversion Mix II.

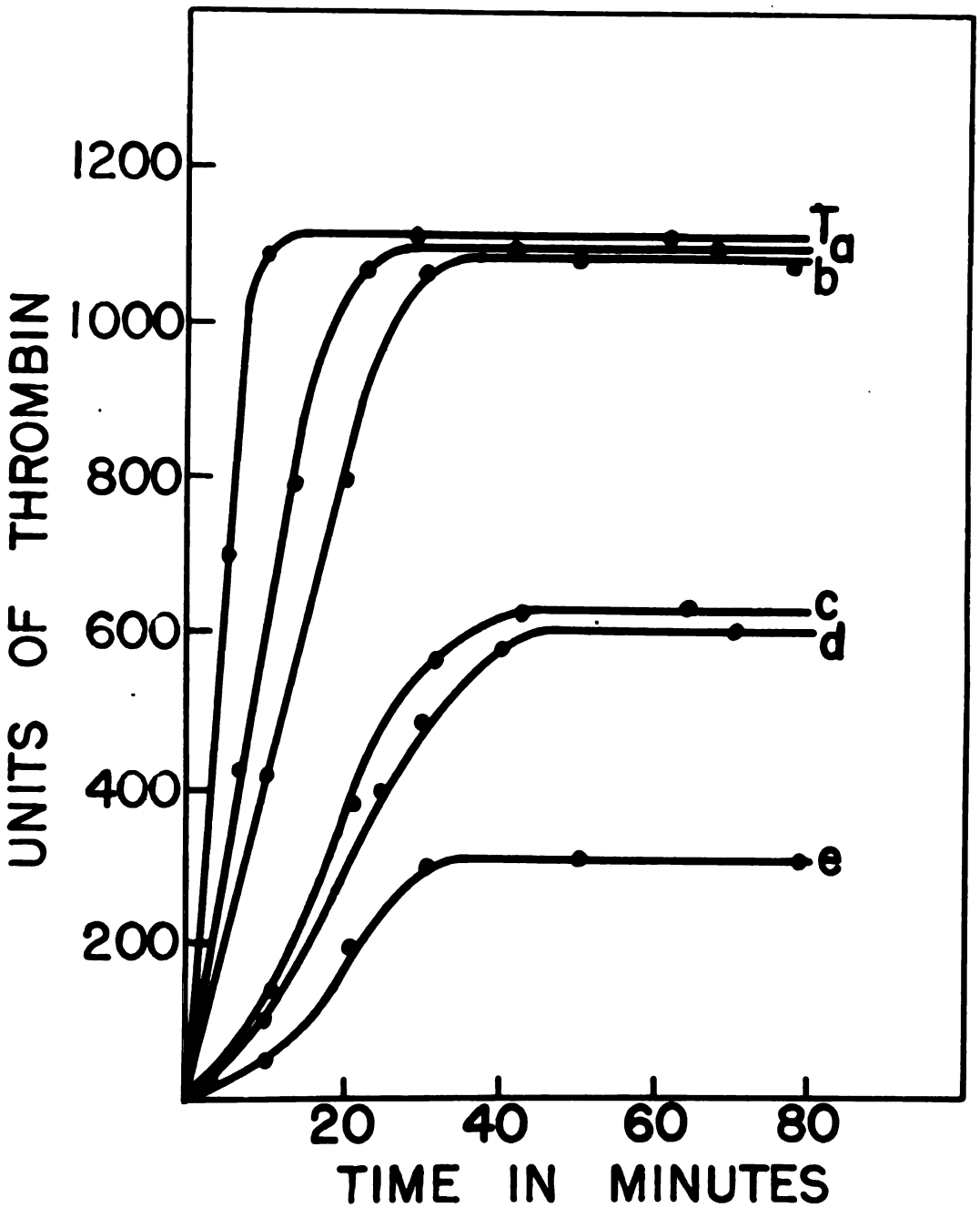


FIG. 8 THE EFFECT OF THROMBIN SEEDING ON THROMBIN YIELD

FIGURE 9

THE EFFECT OF THROMBIN SEEDING ON THE OTHER ACTIVE ANALOGUES OF HISTAMINE AND ON PLATELET EXTRACT CONTROL

Prothrombin 3000 units/ ml.

Organic compounds 1 mg./ml.

Curve A: Lung thromboplastin control.
Conversion Mix I.

Curve B: Decapryn plus 8 units of thrombin.
Conversion Mix III.

Phenindamine plus 8 units of thrombin.
Conversion Mix III.

Benadryl plus 8 units of thrombin.
Conversion Mix III.

Curve C: Phenindamine.
Conversion Mix III.

Decapryn.
Conversion Mix III.

Benadryl.
Conversion Mix III.

Curve D: Platelet extract plus thrombin.
Conversion Mix II.

Curve E: Platelet extract.
Conversion Mix II.

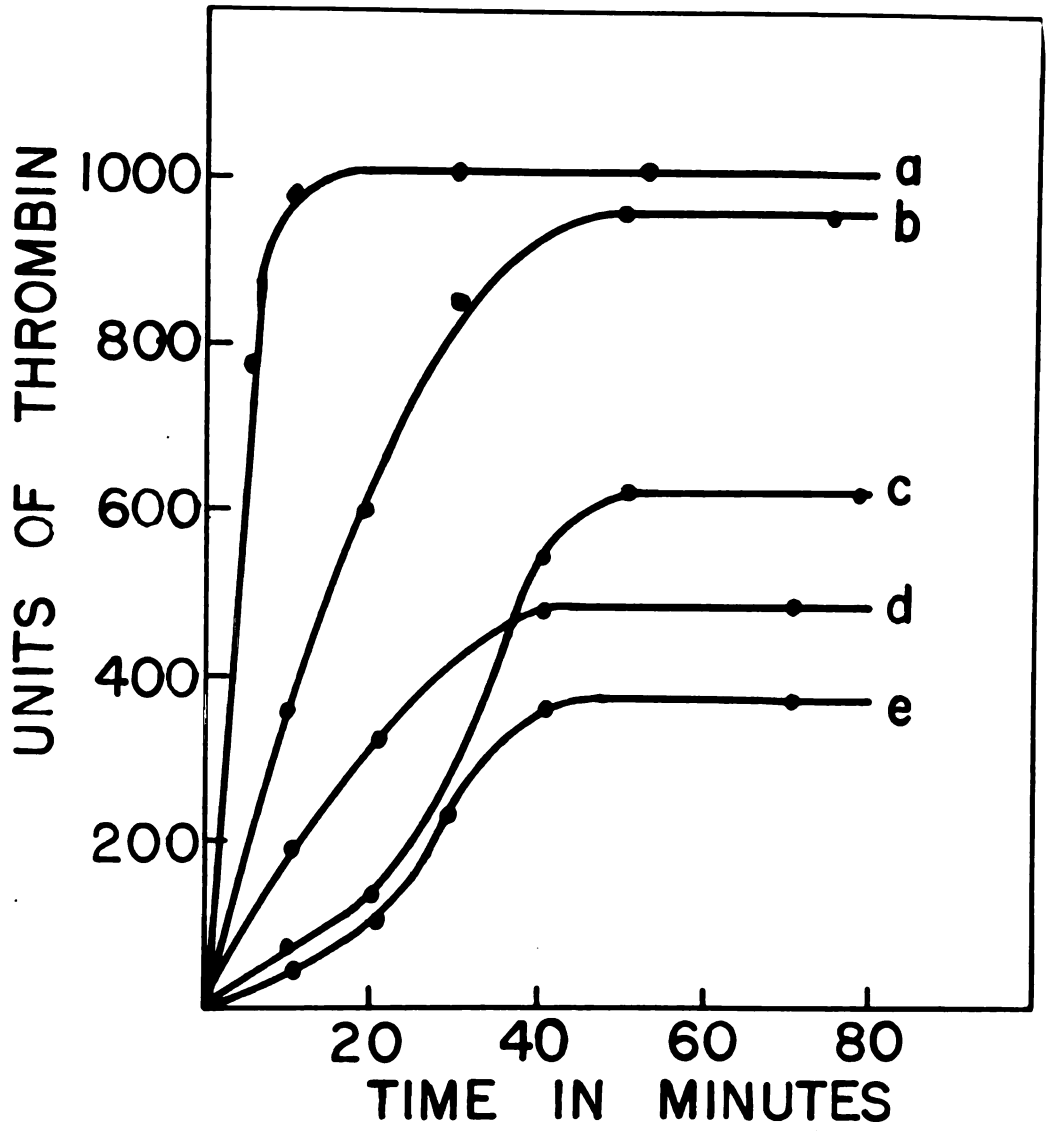


FIG. 9 THE EFFECT OF THROMBIN SEEDING ON THE OTHER ACTIVE ANALOGUES OF HISTAMINE AND ON PLATELET EXTRACT CONTROL

C. QUANTITATIVE RELATIONSHIPS OF THROMBIN SEEDING

The previous experiments indicated that purified thrombin accelerates the conversion of prothrombin to thrombin by some platelet factor - organic activator interaction. Moreover, it has been suggested that this might depend upon the rapid conversion of the plasma Ac-globulin to serum Ac-globulin. The total amount of thrombin used in each assay in the previous seeding experiments was 100 units (concentration 200 units/ml.) or a final dilution of 33 units/ml. of assay material. A series of dilutions of thrombin was prepared. The results are indicated in Figure 9. The minimum amount of thrombin per assay (total 3 ml.) necessary to yield rapid, complete activation of the prothrombin was 4 units per assay (prothrombin 1000 units). It is interesting to note that this quantity is small compared to the amount of prothrombin found in 1 ml. of human plasma (300 units).

It then became necessary to survey those compounds--histamine, benadryl, decapryn, linadryl and phenindamine--which were previously found to be potent activators of prothrombin. The question as to whether the relationship established with linadryl (an active compound picked at random) would hold with the other active compounds. That this is true is readily demonstrated in Figure 10.

In contrast to the results of the survey of the various analogues of histamine, no difference is found in percentage activation of prothrombin by the organic activators when seeded with thrombin. If this is a clue to the mechanism of action of the various components, it can only be assumed that the action of the organic activators must be accompanied by another reaction to complete the activation of the prothrombin present.

FIGURE 10

QUANTITATIVE RELATIONSHIPS IN THROMBIN SEEDING OF ORGANIC ACTIVATORS OF PROTHROMBIN

Prothrombin	1.0 cc.
Platelet extract	.5 cc.
Saline thrombin with X units thrombin/ml.	.5 cc.
Calcium chloride .163 M	.5 cc.
Organic activator	.5 cc.

Curve A: Linadryl plus 16 units/ml. thrombin.

Curve B: Linadryl plus 8 units/ml. thrombin.

Curve C: Linadryl plus 4 units/ml. thrombin.

Curve D: Linadryl plus 2 units/ml. thrombin.

Curve E: Linadryl.

Curve F: Platelet extract.

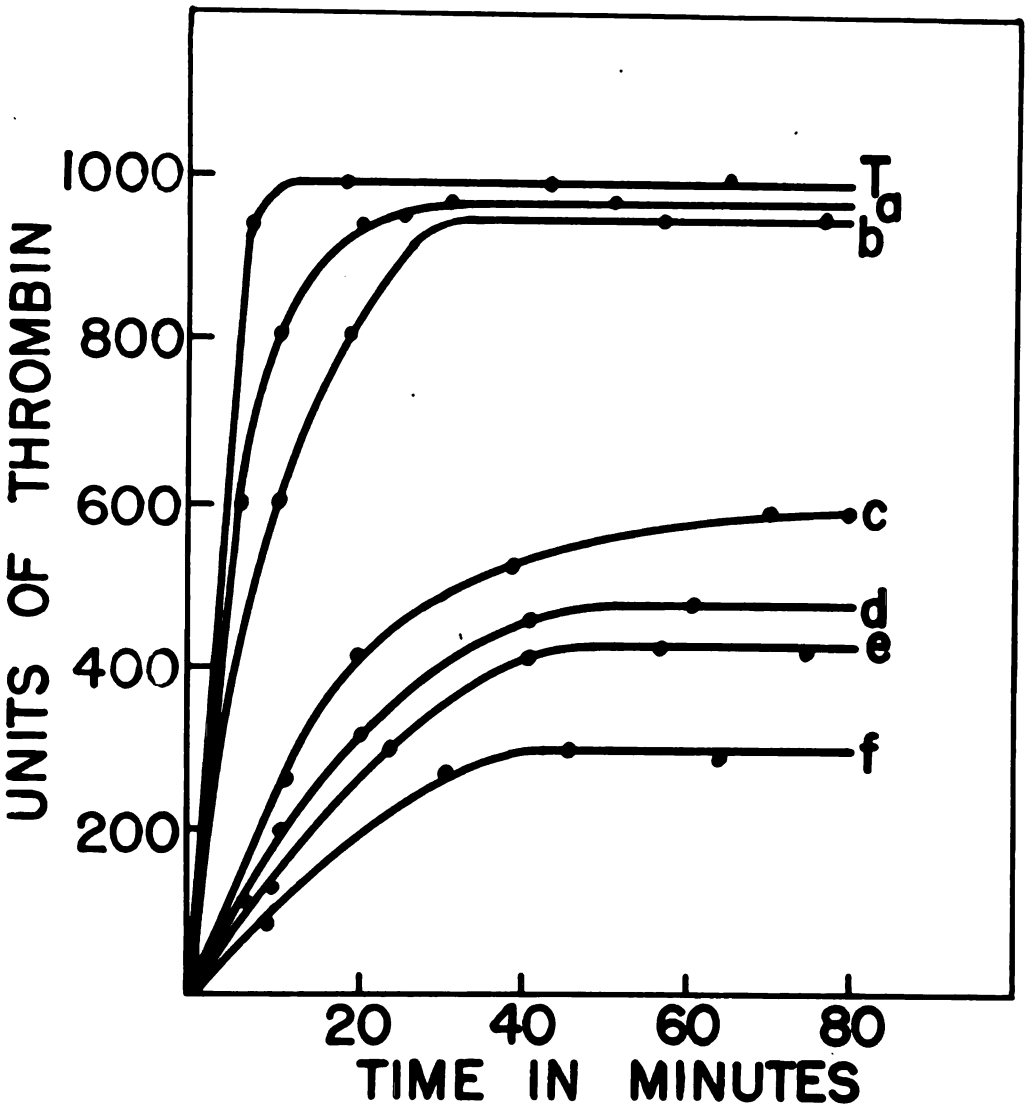


FIG. 10 QUANTITATIVE RELATIONSHIPS IN THROMBIN SEEDING OF ORGANIC ACTIVATORS OF PROTHROMBIN

DISCUSSION

From the outset of the systematic study of the coagulation mechism, organization of the system has required a division of the process into two stages. These were the well known schemes of Morawitz and Howell.

1. Prothrombin $\xrightarrow{\text{thromboplastin}}$ Thrombin

2. Fibrinogen $\xrightarrow{\text{thrombin}}$ Fibrin

Although the entities prothrombin (thrombin) and fibrinogen (fibrin) have been fairly well delineated chemically and in research thinking, our concept of thromboplastin has evolved slowly and the issue is still very much in debate. Early in coagulation work, it was found that body tissue extracts have strong thromboplastic properties. Further studies have shown that brain (Quick), lung and testes were the best sources of this thromboplastic material. Extensive analytic studies, particularly by Chargaff and co-workers[47,48,49] have shown this thromboplastin to consist of various lipoprotein fractions. However, no such comparable compounds have been found in the plasma nor have any compounds been found in the plasma with comparable thromboplastin activity.

Is tissue thromboplastin then physiologic? This question has not been answered to date. Hemostasis can be achieved with tissue thromboplastin. This is one of the bases upon which bleeding time tests rest. Although other variables play a role here, such as surface, vasoconstrictors and platelet emboli, the basic rationale for coagulation time and bleeding time studies is to delineate the intrinsic clotting mechanism of the blood from the total number of hemostatic mechanisms which may well include a tissue thromboplastin.

The nature of the intrinsic thromboplastin mechanism in the blood is now in question. Clinically, two general states occur which give clues to the answer; the thrombocytopenias which are associated with prolonged bleeding times and normal or occasionally prolonged clotting times and the hemophilias which are associated with a prolonged clotting time in the face of normal prothrombin, platelet concentration, Ac-globulin and fibrinogen concentrations. The thromboplastic role of the platelets in initiating hemostasis has been confirmed by VanCreveld[50] and in the isolation of a platelet factor which will act with platelet free plasma to initiate the clotting of the blood. Further experimentation has shown that at least one factor in the plasma which acts as a co-factor for the platelet factor is anti-hemophilic globulin. Let us call this Co-factor I.

We, then, can write the following partial scheme for the initiation of clotting:

7. Prothrombin $\xrightarrow[\text{Ca.}\ \text{Ac-globulin}]{\text{Platelet Factor 3}\ \text{Co-factor I}}$ Thrombin

Recently, investigation of the hemophilia problem has revealed the deficiency of a plasma thromboplastin conversion factor (PTC), as well as of a plasma thromboplastin antecedent factor (PTA). These may very well be the same as Co-factors II and III plus platelet factor 3. If this is the case, the intrinsic thromboplastin mechanisms of blood are complex indeed. Adding also Ac-globulin for rapid activation the scheme might then be enlarged to:

8. Prothrombin $\xrightarrow[\text{Ca.}]{\text{Platelet Factor 3}\ \text{Co-factor I, II, III}}$ Thrombin

Plasma Ac-globulin

Serum Ac-globulin

Recently, Tocantins and co-workers[31,32,33] have shown evidence for a circulating inhibitor of platelet co-factor. Although we recognize the im-

portance of this work we shall not attempt to comment on it at this time.

As described above, various organic compounds related to histamine can substitute for plasma Co-factor I in the activation of purified prothrombin. This is significant. It is difficult to find other cases in which an organic compound will substitute for a protein, with the exception, perhaps, of thyroxine for thyro-globulin. However, inasmuch as the organic activators function erratically in the rapid activation of prothrombin and this activation is often incomplete with reference to the tissue thromboplastin standard, it may well be that what has been identified here represents one or two of the active molecular groups of the Co-factor I molecule.

Compounds which will substitute for Co-factor I are histamine, dimethylamino-ethoxy compounds and a pyridindene derivative.

Of all the synthesized anti-histamine compounds, the great majority are Dimethyl amino ethyl compounds. It is significant that only Oxygen substituted compounds should have thromboplastin activity, except for histamine itself and thephorin. However, the exact nature of this chemical action is still uncertain.

It is well known that the activation of the prothrombin molecule by 25% sodium citrate involves the release of a carbohydrate or glycoprotein residue. An interesting phenomenon in biochemistry is amine catalysis in which primary and secondary amines can split aldol groups under prescribed conditions.[51,52] Although these conditions do not seem to exist in these experiments we should like to call attention to this phenomenon.

Histamine is found in the plasma under physiological conditions. Moreover, histamine is concentrated in the platelets in rabbits and in cells of the myeloid series (Code)[53] in other species. H-substances which may or may not be proteins have been postulated for over 40 years. It may be

that these compounds have a physiological role in initiating the clotting of blood as well as that in relation to release of heparin. The discovery that the analogues of histamine also have thromboplastin properties without the classical effects on the vascular tree and smooth muscle bed, initiates interesting possibilities in relation to therapeutic drugs for the hemophilias.

The nature of the activation curve with the platelet extract and organic activator is an interesting one. Its sigmoidal character indicates a complex of two or more reactions. It is also characteristic of an autocatalytic process, whereas, the hyperbolic curve obtained with lung thromboplastin is indicative of a first order reaction.

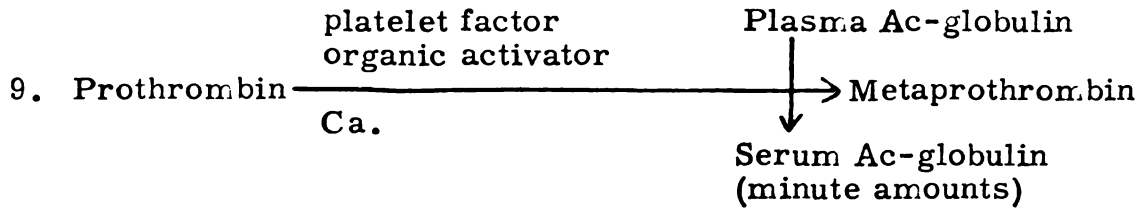
The thrombin seeding experiments assist in elucidating the manner of activation. Inasmuch as thrombin seeding of a purified prothrombin substrate in the absence of other factors results in slow and incomplete activation of the substrate, and inasmuch as we are dealing here with rapid activation of prothrombin, we can say that this does not represent a pure autocatalytic process. Rather, if we should divide the activation curve in two portions, a lag phase and a zone of rapid activation, we could postulate two general reactions. These might well be:

1. The platelet factor organic activator complex reacts with prothrombin to form a metaprothrombin which is easily activated to thrombin.

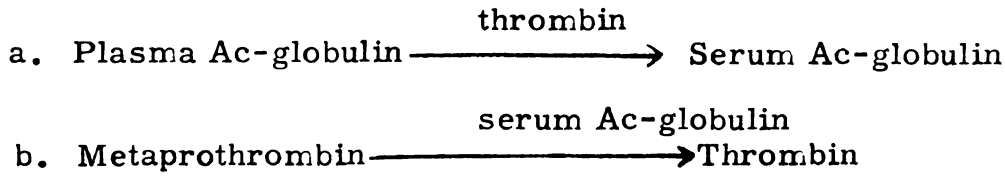
    a. The platelet factor organic activator complex reacts with plasma Ac-globulin--this occurs slowly and in minute amounts.

2. If thrombin is present, plasma Ac-globulin is converted to serum Ac-globulin. Metaprothrombin in the presence of serum Ac-globulin is rapidly converted to thrombin.

This can be represented in the following equations:



10. With the addition of small amounts of thrombin:



The evidence for schemes of this nature is, of course, circumstantial as yet. There are no data presented here which rigorously demonstrate the chemistry suggested. After this is recognized, however, there is still room for a schematic representation upon which to organize future research. It is in this light that the above scheme is presented.

SUMMARY

By means of a thromboplastin assay based on two stage analysis for prothrombin and using purified materials, various organic compounds are surveyed for thromboplastin activity. It is shown that Histamine in conjunction with a platelet factor is capable of activating prothrombin. It is further shown that this "complex" also requires the initial presence of plasma Ac-globulin.

Analogues of histamine are surveyed and it is found that various diethylamino ethoxy compounds and a pyridindene compound possess similar thromboplastin activity. It is found that rapid and complete activation of prothrombin can be achieved by the addition of minute amounts of purified thrombin.

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