"PROTAMINE-HEPARIN TITRATION METHODS FOR THE DETERMINATION OF CIRCULATING ANTICOAGULANTS."

by

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INTRODUCTION

The hemorrhagic syndromes that occurred after atomic bomb irradiation (1) have attracted attention, because the phenomena did not seem to be fully explicable on the basis of thrombocytopenia alone. It was first suggested by Allen and Jacobson (2), that an excess of heparin in the blood might be the cause of the decreased coagulability and bleeding tendency after acute ionizing irradiation. Allen then developed a heparin-protamine titration test (3) for the demonstration of heparin or heparin-like substances in the circulating blood.

In the course of work on blood coagulation it was thought that it would be of interest to evaluate the use of Jaques' heparin-protamine titration method (4) in patients with hemorrhagic tendencies. Furthermore, when Allen had published his method, it seemed of interest to compare the two methods. Thus the present investigation was undertaken.

First, using Jaques' method, heparin-protamine ratios were established and the constancy of the heparin-protamine equivalent and the influence of several factors studied. The method was then applied to the measurement of heparin injected intravenously. An attempt was made to estimate anticoagulant substances in a series of blood dyscrasias. It was found, however, that the protamine titration method was of little value in this respect, but could find another use due to the increased anticoagulant effect of the protamine sulfate in some blood dyscrasias.

Allen's heparin-protamine titration method was tested in normals, and the mechanism of its action elucidated. Modifications of Allen's test were made and compared with Allen's technique in normals, in blood

dyscrasias and in the blood of heparinized and dicumarolized patients.

As a result of the work a hypothesis for the action of "prolonged clotting tests" is offered and their value discussed.

In the first chapters the value and limitations of clotting tests are discussed and the literature on heparin-protamine clotting tests and their application in hemorrhagic diseases is reviewed. A bibliography is included.

C H A P T E R I

The mechanism of coagulation.

It is impossible to review in a limited space the literature on the mechanism of coagulation, and it is not necessary for the purpose of the present investigation. An excellent review is given by MacFarlane (61). Yet, it seems justified to consider briefly the latest views on the subject, as put forward by Seegers (5) in a simplified schedule. He considers the clotting mechanism to consist of two phases, as proposed by Morawitz. (62)

In the first phase prothrombin is being converted to thrombin, which does not occur spontaneously in the blood. This conversion is accelerated by many factors; Calcium ions, thromboplastin, accelerator-globulin (probably identical with Factor V of Owren and the labile factor of Quick), platelet accelerator and possibly other factors (antihemophilic globulin). These factors have a buffering capacity. One factor can compensate for a partial deficiency of another factor (63). The thrombin formed has a twofold purpose: 1) to convert the inactive plasma ac-globulin into active serum ac-globulin. The latter accelerates the first stage of the clotting mechanism, which now proceeds with increasing speed, as an <u>autocatalytic</u> reaction.

2) to convert fibrinogen into fibrin.

So far only accelerators of the clotting mechanism have been mentioned. There are also inhibitors, one of which is <u>antithrombin</u> which inactivates thrombin. Furthermore, there is <u>heparin</u>, which exerts its main function as an inhibitor of the first phase, together with a co-factor in plasma (59). It is not clear whether the action of heparin is antiprothrombic or antithromboplastic, or something

entirely different.

Seegers suggests that the antithrombic action of heparin is in conjunction with crystalline albumin, to promote absorption of thrombin on fibrin. Third, there is antithromboplastin, described as an inhibitor of the first phase of the clotting mechanism.

It is still a problem how the clotting process is initiated. There are two opposite views: Quick (25) believes that platelets liberate an enzyme, which activates thromboplastinogen in plasma to active thromboplastin. Brinkhous (60a) has a different approach. He suggests that a foreign surface factor primarily activates, in the presence of calcium ions, a plasma factor, which becomes a thrombocytolysin, disrupting the platelets to set thromboplastin free. The presence of an inactive thromboplastin precursor in the plasma is also proposed by Conley (60). It seems that platelets, plasma-factor, and foreign surface (or other initiating factor) are necessary, but the pathway of activation is still obscure. The solution of this problem is difficult, because no one has been able, even with silicone technique, to obtain plasma, which is irrefutably free of platelets or platelet products.

Fibrinolysis (5) (dissolution of the fibrin clot) is an enzymatic process that closely resembles the clotting process. Fibrinokinase activates the formation of inactive plasminogen (profibrinolysin) into active plasmin (fibrinolysin), which dissolves the fibrin clot. Plasmin is inactivated by antiplasmin. Antiplasmic activity is also exerted by heparin.

C H A P T E R II

Clotting tests :

It seems appropriate to evaluate clotting tests in general before dealing with special clotting methods.

(1) Introduction :

In vitro clotting tests on whole venous blood have been used for a long time in the diagnosis of hemophilia. It was realized that delayed coagulability of the blood in vitro had something to do with a bleeding tendency, and thus it was assumed that in vitro coagulability of the blood paralleled in vivo coagulability. But it soon became apparent that the in vivo coagulability was not the only factor that influenced the clotting time in vitro. Consequently a variety of clotting tests have been developed which tried to avoid environmental errors that might affect the method.

If one realizes that coagulation is a biochemical, probably enzymatic (5) process, it is obvious that any attempt to get information about it, by measuring the speed with which the process evolves, is an extremely poor one. Biochemical processes are influenced by many environmental factors (for instance temperature, p_H), that may affect their rapidity. However, the fact that even today clotting times are done faithfully to obtain information about the coagulation mechanism can be explained on the one hand by the misleading simplicity of some of the methods used, and on the other hand by the time consuming and costly methods that are required for fractionation of the different protein factors involved in the clotting process, needing specialized biochemists, technicians and laboratory outfit. These methods are not suitable for routine use. Therefore, clotting studies are still being done, and for their evaluation it is worthwhile to consider the in vitro factors, that may influence the clotting time of whole venous blood. These factors may be considered as

so many errors that may spoil the method, the ultimate purpose of which is to get information about the in vivo fluidity of the blood.

(2) Factors, other than in vivo coagulability, that influence the clotting time of whole venous blood: (6, 7, 8)

1) Admixture of traces of tissue juice shortens the clotting time tremendously. Thromboplastin, present in tissue juice, is responsible for this effect. Clumsy venapuncture, use of a dull needle and interrupted blood flow during blood withdrawal tend to shorten the clotting time. Choice of a superficial vein is preferable.

2) Temperature: It has been demonstrated that elevation of the temperature shortens the clotting time whereas a low temperature prolongs the clotting time. Clotting times have to be performed in a constant temperature waterbath (usually 37°C).

3) Contact with foreign surface (9): a) Type of foreign surface:- Contact with glass markedly shortens the clotting time. Therefore, other substances have been used to avoid the accelerating influence of a glass surface, for instance paraffin (10), lusteroid (11,12), collodion (6), silicone (13,14,15) and polyethylene (16). Clotting times of blood in these non water-wettable materials are relatively prolonged and thus are believed to give better information on the in vivo coagulability and to demonstrate smaller changes of the in vivo coagulability of the blood. The influence of Zeta-potentials (9,17) in relation to surface phenomena in blood clotting is under investigation.

b) Consequently, if the type of foreign surface is of importance, the extent of the contact area is important. Clotting times have to be done in standardized tubes, using standard size needles, syringes and a standard volume of blood.

c) Cleanliness: Unevenness of the contact surface, dust, traces of old blood, and traces of acid (18, 18a) tend to shorten the clotting time (the latter probably due to p_H changes).

4) Contact with air: In a tube containing blood, the first fibrin thread always appears at the air-glass-blood interface. Contact with air shortens the clotting time. So do air bubbles or foam in the syringes and the tubes. A standard tube diameter is also of importance in this connection.

5) Movement: A wide angle of tilting of the tube, frequent tilting and axial rotation all shorten the clotting time (mixing effect).

6) Method of timing (starting of the stopwatch) and reading of the endpoint should be rigidly standardized. In a glass tube the endpoint is usually taken as the time at which the tube may be inverted without spilling blood. However at that time the blood is not totally clotted. The phenomenon simply depends on the strength of the fibrin sheet that covers the fluid blood.

7) p_H changes: Extremely high or low p_H values inhibit coagulation in vitro.(8)

(3) Conclusions drawn from the above list of factors influencing the in vitro clotting time:

1) All clotting tests should be rigidly standardized.

2) Normal values, range of variation and standard deviation should be established, if possible, before conclusions concerning significant shortening or lengthening are made.

3) Clotting times on finger blood are of no value, as the blood so obtained contains a variable amount of tissue juice, the quality of which may be influenced by factors unknown (for instance by the state of hydration of the patient).

4) Most in vitro factors (except extreme p_H values, low temperature and the use of water-repellent tubes) have a shortening effect on the clotting time. Therefore, a shortened clotting time in a glass tube cannot be readily accepted as the reflection of an increased in vivo coagulability. The finding of a prolonged clotting time is of much greater importance. Errors in technique may shorten a prolonged coagulation time to an apparently normal value.

(4) Silicone technique:

The use of silicone coated glassware eliminates the accelerating effect of glass on the clotting time. For a proper technique, needles, syringes and tubes should be coated with the silicone, and two syringes used. (19) The blood in the first syringe is discarded. The blood in the second syringe is used for the clotting time determination, after discarding the first 0.5 cc. This method rules out most of the variations due to admixture of traces of tissue juice, which cannot be avoided by vena puncture. With this technique smaller changes in the in vivo coagulability of the blood due to acceleration or prolongation are indeed demonstrable, but unfortunately the method is also more sensitive to technical errors. Shortening of clotting times in siliconed tubes has to be interpreted very critically. (6,20) before conclusions regarding increased in vivo coagulability are made.

(5) What information may be obtained from a properly performed clotting test, and how should the result be interpreted?

The clotting time may be shortened, prolonged or normal reflecting increased, decreased or normal in vivo coagulability.

Theoretically, increased coagulability may be due to:

1) excess of accelerators.
2) deficient inhibitors.

decreased coagulability may be due to 1) excess of inhibitors.

2) deficient accelerators.

A normal clotting time, however, does not necessarily have to be the reflection of a normal constitution of the blood in accelerators and decelerators. Lack in accelerating factors may be masked by the clot accelerating effect of the glass wall, but also, as has been pointed out by Seegers (5), (and as is well known in the case of hypoprothrombinemia), there is a great buffering capacity in the clotting mechanism itself due to the variety of accelerating factors.

Little is yet known about inhibitor deficiencies.

An in vitro clotting time may also be normal in the presence of small amounts of added inhibitor (for instance heparin), due to the glass wall effect. As has been pointed out above silicone clotting times are more sensitive in this respect.

(6) Classification of increased or decreased in vivo coagulability:

a) Increased coagulability:	In thrombosis.	Due to increased thrombo-plastin in circulating blood?
		Other possible causes still hypothetical.
b) decreased coagulability: due to		
(1) Deficiency in a clotting factor or accelerator factor:		(Hemophilia (antihemophilic (globulin lacking? (21).
		(Fibrinogenopenia (fibrinogen (deficiency).
		(Prothrombin deficiency (usually (not demonstrable in a glass tube).
		(Ac-globulin deficiency (= Factor (V deficiency) (22).
		(Platelet deficiency (lack of (platelet accelerator) (23). (Demonstrable in silicone tubes ((8) and in heparin clotting (test (36) and prothrombin (utilization rate (57).

(2) excess of inhibiting factor:
- antithrombin excess.
- heparin excess (acts mainly as antiprothrombin). Proven in anaphylactic shock in the dog.(4)
- heparin-like substances (hypothetical). (58)
- Thromboplastin inhibitor (24).
- Antithromboplastinogen (25,26).
- Circulating anticoagulant in hemophilia (antibody type) (27,28).
- Circulating anticoagulants, not further identified.

Some of the substances under the group of excess inhibitors are still hypothetical, and their differential diagnosis is not satisfactorily worked out.

(7) "Prolonged Clotting Time Methods":

As has been pointed out, the ordinary glass tube clotting time method of Lee and White (29) is not sensitive enough to diagnose small changes in blood coagulability. Lately so called "prolonged clotting time methods" have been developed for this purpose. They are:

1) the "silicone clotting time", which has been described above, by which the clot accelerating effect of glass is avoided.

2) the clotting time, which is obtained by addition of a standard amount of blood to a small amount of an anticoagulant in a glass tube. The anticoagulant used in most cases is heparin, which gives a prolonged clotting time, due in part to the fact, that heparin nullifies the accelerating effect of the glass wall. There are a variety of heparin clotting tests:

a) The Waugh-Ruddick test: (30,31,32) The authors developed the test to show the presence of increased coagulability, which could not be detected by the Lee-White method. In this test 1 cc. of venous

blood is added to each of a series of tubes containing increasing amounts (1-7 gamma) of heparin in 0.5 cc. of saline. The clotting times were originally determined at room temperature, later at a constant temperature of 37°C. (32). The clotting times plotted on the ordinate against the heparin concentrations on the abscissa give a curve with a certain slope. The curve shifts clockwise or counter-clockwise with increasing or decreasing coagulability of the blood.

b) The Waugh-Ruddick-Silverman test (33) is a modification, using recalcified oxalated plasma and a waterbath at 37°C.

c) Hagedorn (34), Rosenbaum and Barker (35) and later Rosenthal (36,37) simplified the test by using only one tube with a standard amount of heparin. Rosenthal uses 4 gamma of heparin in 0.1 cc. of normal saline to which 1 cc. of blood is added. The test is carried out at 37°C.

RESULTS: These tests are subject to any error which may affect coagulation tests. Foaming (38), temperature (32), clumsy venapuncture etc. influence the outcome. Increased coagulability (flattening of the curve) has been found when thromboplastin was added to the tubes, and during bedrest in acute infections, in the postoperative period and after hemorrhage (30,31), venous thrombosis and embolism (35) and following coronary thrombosis (38).

The Silverman modification showed increased coagulability in postoperative cases (33) when used by the author. Others (36,39,40) could not obtain constant results with this technique.

Decreased coagulability (steepening of the curve) was found by Waugh and Ruddick in radium irradiated cases, and when the platelets were reduced. (30,31). Rosenthal (36,37) found a prolonged heparin clotting time in 11 out of 21 cases of leucemia (8 of the 11 had platelet counts below 200,000.) In polycythemia vera the heparin clotting time became normal or rapid when the results were corrected

for the hematocrit increase. (Most of these patients had a high platelet count.) An increased susceptibility of thrombocytopenic blood to heparin had been suggested first by Allen (43). Lyle and Fowler (41) clearly demonstrated the influence of thrombocytopenia on the Waugh-Ruddick tests, in which the blood became incoagulable in the tubes containing 3 gamma or 4 gamma of heparin or more. Conley (42) also demonstrated, using silicone technique, a direct relationship between the concentration of heparin required to inhibit the coagulation and the number of platelets. The influence of the platelet count on the clotting time of heparinized blood thus seems established.

In vivo heparin tolerance tests have been carried out (44,45) but will not be discussed here.

d) Instead of heparin a different anticoagulant could be used. Such a test, with protamine sulfate, in increasing amounts in a series of tubes, or in only one tube will be dealt with in the chapter on experimental work.

(8) Special techniques for the demonstration of circulating anticoagulants :

These are numerous, because there is such a great variety of anticoagulants possible. Most tests are described in a recent article by Dreskin and Rosenthal (26).

a) A simple technique for the demonstration of an anticoagulant, and also a differential test between anticoagulant and deficiency in a clotting factor, is the addition of increasing small amounts of the pathological blood specimen to normal blood, either in glass tubes (8,26) or in silicone tubes (19.) Prolongation of the clotting time of the normal blood by addition of the abnormal blood may lead to the conclusion that an inhibitor is present in the

pathological blood specimen. In case of a deficiency in one of the clotting factors in the abnormal blood, the clotting time of the normal blood does not become prolonged. Furthermore, the addition of small amounts of normal blood to the abnormal blood will reduce the clotting time of the abnormal blood to normal. If there is both a lack of some substance and an anticoagulant (as in the case of hemophilia with circulating anticoagulant (27,28), the abnormal blood will prolong the clotting time of normal blood.

This test informs about the presence of an anticoagulant but not about its nature and site of action in the clotting mechanism. For this special tests are required.

b) Special tests for anticoagulant activity:

(1) Test for antithrombin activity (47,48)

(2) Precipitin test for antibody formation against antihemophilic globulin (27,28).

(3) Antifibrinogen activity may be ruled out by titrating patients plasma with increasing dilutions of thrombin (26)

(4) Antiprothrombin activity can probably be ruled out with the dilute prothrombin time method using barium sulfate treated, prothrombin free plasma (49).

(5) Antithromboplastin activity may be demonstrated by titration of the patients' plasma and normal plasma against dilute thromboplastin solutions and comparison of the results. (24,26).

(6) Antithromboplastinogen activity can be shown only after excluding all other possibilities. (25,26).

c) The demonstration of heparin may be classified as follows:

1) the chemical purification of heparin, for which there are several techniques. (The names of Howell, Charles and Scott, Fischer, Jorpes are connected with the different methods (46).)

The only one of importance at the present time for clinical use is Jaques' method using N-octylamine (50,51,52). Even this method, however, requires rather large amounts of blood, and is not suitable for routine use.

2) Heparin <u>assay</u> methods, using the anticoagulant power of heparin, (46,53,54,55,56). These methods are not useful, if one is not certain about the nature of the anticoagulant material in the first place.

3) <u>Heparin-protamine titration tests</u>. These will be reviewed in the next chapter.

CHAPTER III

Heparin and its interactions with protamine and toluidine blue.

The chemical and physiological properties of heparin, protamine and toluidine blue are described first.

(1) Heparin:

Heparin (46,59,90,91) is an acid material, which is available commercially as the sodium salt. It was discovered by MacLean in liver extracts. Howell, Best, Charles and Scott, and Jorpes have done much for the purification of the material and for the identification of the chemical structure.

Heparin is a mucoitin polysulfuric ester (64,65,66) of high molecular weight, and belongs to the same group as chondroitin sulfuric acid and mucoitin sulfuric acid. In contrast to the latter substance, heparin contains not one, but several sulfuric acid groups for each glucosamine-glucuronic acid group. Jorpes prepared a mucoitin tri-sulfuric acid, and probably many of the preparations contain mixtures of di- and tri-sulfuric acids. The sulfuric acid group is essential for the anti-coagulant activity of heparin and many other substances with a high molecular weight.

Due to the strongly acid properties heparin forms salts with many basic substances (78), sodium, barium, brucine, protamine, toluidine blue, histones, hemoglobin, and with other proteins (79,90). The combination of heparin with toluidine blue gives a color change from blue to reddish purple, which is called metachromasia. Many sulfuric acid esters of high molecular weight may give this color change, but the strongest color reaction is obtained by heparin (70). It is this property of heparin, that led Jorpes, Holmgren and Wålander to the discovery of heparin in the mast cells of Ehrlich, a discovery which found ample confirmation in the fact that after

anaphylactic shock in the dog the mast cells in the liver are devoid of their staining granules and heparin can be extracted from the incoagulable blood (4). The location of the mast cells along the capillaries suggests a physiologic function to maintain blood fluidity. Increased content of mast cells in the tissues is found after atomic bomb irradiation (1), and also in mast cell tumors in the dog (71). In the latter no prolongation of the coagulation time has been demonstrated. The mechanism of the liberation of heparin from the mast cells is as yet unknown.

The anticoagulant effect of heparin injections is temporary. The effect wears off soon, due to the conversion of heparin, by heparinase, to uroheparin, a degradation product with less anti-coagulant activity that is excreted by the kidneys (91). Excessive amounts of administeréd heparin are rapidly excreted in the urine (72,73,124). Normal human blood contains approx. 0.1 gamma of heparin/cc.(51)

Actien on the coagulation mechanism: 1) Heparin inhibits the first clotting phase, in combination with a cofactor (68,69,75,76), present in the crude albumin fraction, which is not albumin itself. Large doses of heparin prolong the prothrombin time (74). How heparin retards the conversion of prothrombin into hrombin, whether it is mainly an antithromboplastic (77,123) or an antiprothrombic action, or both, is not clear. An excess of thromboplastin will shorten the clotting time prolonged by heparin (30,31), and, vice versa, heparin will prolong the clotting time shortened by thrombo-plastin (74).

2) The antithrombic action ofheparin is now believed to be less important. Heparin is not identical with antithrombin (47,59), but acts, probably in connection with crystalline albumin, to promote absorption of thrombin on fibrin (5).

Intravenous injections of heparin give a maximum prolongation of the clotting time after a lag period of 10 minutes (67). The anticoagulant effect of heparin is more pronounced in vivo than in vitro. These facts and the increased anticoagulant activity after incubation in vitro point to the probability that heparin has to combine with the components of the clotting system before it can exert its maximal effect. (67)

3) Heparin exerts an inhibiting effect on plasmin (80).

4) Influence on platelets: Solandt and Best first reported that heparin in large doses prevented agglutination of platelets in vitro and in vivo.(81) Later it was noted that, after intravenous injection of smaller doses in dogs, a temporary thrombocytopenia and agglutination of platelets was a constant finding (83,84). In other species the findings were not constant.(82) They were sometimes accompanied by emboli and petechiae (85,86). However, thrombocytopenia and clumping of platelets is not a constant finding after intravenous injections of heparin in man (84,83,87).

5) Influence on the clotting time: Heparin prolongs the clotting time in vitro in such a way, that a linear relationship is found between heparin dosage and the logarithm of the clotting time obtained. This was first demonstrated by Fischer and Smitz (88), and confirmed by Bertrand and Quivy, (89) and Jaques.(67) The formula for such a relationship is:

$\log T_2 - \log T_1 = k(c_2 - c_1)$, in which T_1 and T_2 are clotting times, c_1 and c_2 are heparin concentrations at two points on the line, and k is a constant, determined by the angle of the line with the abscissa. The value of k depends on (1) circumstances under which the test is performed, and (2) anticoagulant potency of the heparin preparation used. If these factors are kept constant, k forms the

"heparin sensitivity value" of Jaques. If the heparin concentrations are chosen so that $c_2-c_1=1$, the heparin sensitivity of a certain blood specimen under constant conditions can be calculated. As Jaques has pointed out, another method to determine the heparin sensitivity is to report the clotting time in a single tube containing heparin, but this method is not as sensitive. The Fischer-Smitz formula is applicable to many anticoagulants (89). It should be stressed that the effect of heparin on the clotting time is influenced by all *in vitro* factors, that influence any clotting test (Chapter II) in addition to *in vivo* factors (variation between species and between individuals, anaesthesia, dicumarolisation, etc.) (52).

(2) Protamine sulfate used in these experiments is the sulfate salt of salmine, a protein derived from the testis of the salmon, with a molecular weight of approximately 8000. Its basic properties are due to the large content (87.5%) of arginine (92). Its main use is to prolong the action of insulin in the treatment of diabetes.

Protamine is destroyed in the blood, probably by the enzyme protaminase (93,94). Toxic effects in animals have been observed after intravenous administration of large quantities (95,96,99) partly due to intra vascular occlusion (97,98). These symptoms were absent when smaller doses were injected slowly (99,100,101,102). In man doses up to 100 mgm. have been given with safety (103,125). Extensive investigations have proven it to be non antigenic (104,105), probably due to the lack of an aromatic group (105), but two contrary reports have appeared in the literature (106,107).

Action on the clotting mechanism: Protamine prolongs the clotting time in vitro and in vivo (96), and Chargeff and Olson showed that it neutralizes the anticoagulant effect of heparin in vitro and in vivo

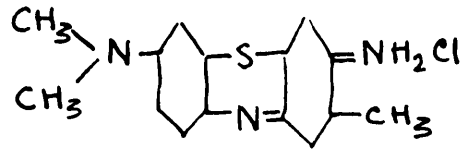
(108). If heparin is injected one hour after the protamine, the neutralizing effect is not observed (109). Subcutaneous injection of protamine has no influence on the clotting time (108). Portman and Holden could not demonstrate an anticoagulant effect on the clotting time after intravenous injection of 100 mgm. protamine sulfate (103) in humans.

The action of protamine on the clotting mechanism may be summarized as follows:

1) Interference with the conversion of prothrombin to thrombin (110,103), which may be overcome with excess of thromboplastin. This has been called antiprothrombin activity, but the exact mechanism cannot be further clarified unless purified prothrombin is used.
2) An antithrombin activity has been mentioned (111), but is not satisfactorily demonstrated.
3) Protamine combines with fibrinogen (103), forming a precipitate in high concentrations (1.5 mgm. per cc. plasma). This is called fibrinoplastic action by Ferguson (112,110).
4) Protamine combines with cephalin (true salt formation) (113,114,115). This action is called antithromboplastic by Tocantins (116).
5) Protamine combines with heparin in true salt formation.
6) It promotes fibrinogenolysis (112).
7) It does not neutralize the heparin cofactor (103).

In experimental thrombocytopenic purpura in animals, protamine reduced the petechiae by 90% without influencing the platelet count (117).

(3) Toluidine blue (118) is a dye with basic properties. It belongs to the group of thiazins, which are characterized by an -N= group and an S- atom attached to the phenyl and quinone group of the molecule. Methylene blue and thionine are other members of the thiazin group. The formula of toluidine blue O is:



It has an anticoagulant effect in vitro and in vivo and neutralizes the anticoagulant action of heparin in vivo and in vitro in much the same way that protamine does (119,127).

Toxic effects occurred in mice that ingested large doses of toluidine blue (anemia, and siderosis of organs) (120). In man nausea and vomiting have been observed (121), when the dye was given in doses of 2-10 mgm. per KG. body weight. Large amounts of toluidine blue (more than 2 mgm. per cc) added in vitro to oxalated plasma was reported to cause "coagulation" without addition of calcium (122), but this may well have been due to fibrinogen precipitation.

The metachromatic color change that occurs when toluidine blue combines with heparin is used for heparin determination (70,73,52, 126).

(4) Reactions of heparin with proteins and complex bases:

Fischer (79) observed that heparin forms compounds with proteins, which are true salts. Heparin thereby changes the properties of the protein (isoelectric point, solubility). This relationship between heparin and proteins is stoichiometric and reversible. Dissociation of the compounds obeys the mass law (78).

Thus :

protein + heparin = protein-heparin.

$$\frac{C_{protein} \times C_{heparin}}{C_{protein\text{-}heparin}} = K$$

(C= concentration
K= constant)

Jaques (78) investigated the subject thoroughly and confirmed this relationship for the interactions between heparin and many basic substances and proteins. The compounds behave as simple salt formations. Examples are the compounds between :

heparin and protamine

histone (79)

toluidine blue

thromboplastin (protein part) (77,79)

proteins (79), for instance hemoglobin, casein, gelatin.

cephalin and protamine (113,114,115).

histone (115)

thionin (115)

nucleic acid and clupein (79).

Due to the anticoagulant properties of heparin, this type of reaction has become important in coagulation studies. Protamine combines with heparin and by doing so, neutralizes the anticoagulant activity of heparin (79,108). The same has been shown for toluidine blue, histone and thromboplastin.

The heparin-protamine titration method :

Jaques developed a method for the estimation of small amounts of heparin in blood (99). By adding samples of heparinized blood to a series of tubes containing increasing amounts of protamine sulfate, the clotting time in these tubes first decreases with increasing amounts of protamin until it reaches the normal value found in the

absence of heparin and then increases again. Plotting of the clotting times against protamine concentrations gives a V-shaped curve. The clotting time is prolonged in the presence of excess heparin or protamine and it is normal at the equivalence point, where the heparin activity is completely neutralized by protamine. It has been shown, that at the equivalence point the amount of protamine necessary to completely suppress the anticoagulant activity of a certain amount of heparin is constant, but it may be different for different lots of protamine and heparin.

The amount of protamine, which suppresses the heparin activity completely at the equivalence point, is not the same as the amount of protamine actually bound by heparin, but is greater. According to Jaques this is the reason why the equivalence point is distinct, showing a sharp angle between the two limbs of the V-shaped curve. It explains why small amounts of added protamine now give a demonstrable anticoagulant activity.

The reaction between heparin and basic substances is influenced by many factors: 1) changes in p_H, 2) addition of anions and cations, 3) addition of protein, 4) addition of lipoid.

Increase in p_H gives an increase in the dissociation constant of the heparin-base compound, ultimately leading to complete hydrolysis. Addition of anions and cations also results in dissociation of these compounds, since they compete for the base and the heparin respectively (addition of NaCl and $CaCl_2$ increased the dissociation constant). Thus addition of anions will decrease the amount of heparin bound to protamine by combining with the protamine, and cations will decrease the amount of heparin bound to protamine by combining with the heparin. By addition of proteins to a system containing heparin and protamine, heparin will

distribute itself between these two basic complexes according to their relative concentrations and to the dissociation constants of their heparin compounds.

Lipoid (78) on the contrary will increase the binding power of proteins to heparin.

These facts are of importance in heparin-protaminetitration methods for the estimation of anticoagulant activity.

The well known coagulant effect of dilution on peptone plasma (62) is explained by Jaques as a dissociation phenomenon (78). The influence of serum dilutions on heparin-toluidine blue mixtures is shown by Seitz (134), who also developed a method for the determination of total serum protein by heparin titration (135).

(5) Heparin-protamine titration methods :

a) Heparin-protamine titration method (Jaques) : (99,78).

A series of tubes, containing increasing amounts (1 to 100 gamma) of protamine sulfate in 0.5 cc. saline is set up and 0.5 cc of the blood, which is to be investigated for heparin content, is added to each tube. The contents of the tubes are mixed and the clotting times are determined in a waterbath at 37°C. The shortest clotting time in the series indicates the equivalence point. If the heparin equivalence for the protamine solution is known, the heparin content of the blood specimen can be calculated in terms of the heparin standard used.

The heparin-protamine ratio for a certain protamine solution can be found by placing 0.50 cc. of normal blood in each of a series of tubes containing a standard amount of heparin, and increasing amounts of protamine. The shortest clotting time in the series will give the heparin-protamine equivalent for the solutions used.

b) Instead of protamine sulfate other investigators have used toluidine blue (133). Haley and Stolarsky (127) foundtoluidine blue, Azure A, Neutral Violet and Neutral Red of equal value.

c) Heparin-protamine titration method (Allen): (3,129).

Venous blood is put in a centrifuge tube to the 11 cc. mark and mixed with 1 mgm. of heparin, so that each one cc. contains 90 gamma of heparin. One cc. samples of this heparinized blood are then added to test tubes containing increasing amounts (100 to 200 gamma) of protamine sulfate in 0.1 cc. of distilled water. The contents of the tubes are mixed, allowed to stand at room temperature and investigated for clotting after one hour. The tube with the smallest amount of protamine which contains a solid clot after one hour, is said to contain the equivalent of 90 gamma of heparin. The heparin-protamine equivalent shows a shift to the right, if a larger amount of protamine is required to give a clot after 60 minutes. Allen found the equivalent to be at 140 gamma of protamine. The equivalent has to be determined with each new stock of solutions used.

Errors affecting the method are: dirty equipment, poor venapuncture, inaccurate measurement of fluids, deterioration of solutions, inadequate mixing, gelation of the blood instead of clotting.

Contrary to a suggestion made by Conley (42), the protamine-heparin titration test of Allen, is, according to the author, not affected by a low platelet count (130), although the test may be positive in cases of thrombocytopenia, and, although the author states that thrombocytopenic (and also prothrombin deficient bloods) are more sensitive to added heparin both in vivo and in vitro. Only a prothrombin activity of less than 25% of the normal will

affect the outcome of the test. The test is curiously not affected by intravenous administration of heparin in amounts up to 50 milligrams.

The author concludes, however, that the test is affected by any factor that prolongs the whole blood clotting time, and thus is positive in hemophilia and afibrinogenemia, and may be positive, if a prothrombin deficiency is complicated by heparinization.

The conclusions drawn from a positive test have varied. At first the presence of an anticoagulant (heparin or heparinlike) was supposed to be responsible for a shift in the protamine titration in this test. Later the conclusions became less specific so that finally the test seemed to indicate only a decreased coagulability similar to, but not identical with, a clotting defect produced by the administration of commercial heparin (58).

As can be seen, the method of Allen differs from Jaques' method in the following points:

1) the test is performed at room temperature.

2) a large amount of heparin is added to the blood.

3) relatively large amounts of protamine are used.

4) the solution of protamine is made up in distilled water instead of saline.

5) the endpoint is read at a fixed time: one hour.

d) <u>Heparin-protamine titration method (LeRoy)</u>: (131,132)

In principle this is the one stage prothrombin time applied to Allen's test. Oxalated plasma is used, and 0.1 cc. is added to each of a series of tubes, each containing 0.1 cc. of a heparin solution of standard strength (10 or 20 gamma per tube), plus 0.1 cc. of a protamine solution containing increasing amounts of protamine (10, 15,20,30,40 or 50 gamma per tube). To this mixture 0.1 cc. of a

thromboplastin-$CaCl_2$ solution is added and the clotting time determined at 37° (in seconds). The heparin-protamine ratio is found at 1:1.5. The method is sensitive enough to demonstrate 3.3 gamma of heparin per cc. of oxalated plasma. It is of interest that administered commercial heparin can be shown with the protamine titration alone (without the addition of heparin to the tubes), whereas in blooddyscrasias it is "generally necessary to perform the complete heparin-protamine titration test". An increased protamine-heparin equivalent is interpreted as due to hyperheparinemia. It can be observed, that in this test the clotting times at the equivalent point are always a few or several seconds longer than the control clotting time.

CHAPTER IV

Application of the heparin-protamine titration methods in hemorrhagic diatheses due to circulating anticoagulants.

Definition of a circulating anticoagulant: This is a substance in the blood stream, which inhibits coagulation in vivo. If it occurs in excess, it may give rise to a hemorrhagic tendency, and is then demonstrable in vitro by special clotting tests.

Very little is known about the nature and variety of circulating anticoagulants. Theoretically, every inhibitor of the clotting mechanism, when it occurs in excess, may give rise to hypocoagulability. Therefore, for the classification of hypothetical circulating anticoagulants, the classification of inhibitors of the clotting system is used (see Chapter II, page 8). These are: antithrombin, heparin, heparin-like substances, antithromboplastin (or thromboplastin inhibitor), antithromboplastinogen, circulating anticoagulant in hemophilia (anti-antihemophilic globulin), and circulating anticoagulants not further identified.

A classification of the hemorrhagic diatheses, proven to be or supposedly due to the presence of a circulating anticoagulant, is given in table I. They have been divided into 5 groups.

Group I holds those cases, which are scattered through the literature of the last 12 years, recently reviewed by Dreskin and Rosenthal (26). Some occurred in known hemophiliacs (28, 138, 139, 141,147,149), others were not connected with this disease (25,26,136, 137,140,148,149). In some hemophiliacs it was possible to identify the anticoagulant as an antibody against the factor (antihemophilic globulin), which is lacking in this disease (28,139). It was asscciated with gamma globulin in three cases (28,139,142).

TABLE I

Group:	Hemorrhagic diatheses, proven to be or supposedly due to circulating anticoagulants:	Nature of anticoagulants	Demonstration	Clotting Time
1	a) Hemophilia, after repeated transfusions. b) Hemophilia-like disease 1) in women, after pregnancy 2) in men and women, cause unknown.	a) antibody 1) probaby antibody 2) antithrombin antithrombo-plastin antithrombo-plastinogen	Prolongs C.T. of normal blood	Markedly prolonged.
2	Secondary to: a) Irradiation: 1) man (A-bomb). 2) Exp. animals (A-bomb) 3) Exp. animals (total X-irradiation) (dogs)	Unknown Heparin ? Heparin ?	1) Not done 2) Neutralized by protamine 3) Chemical + Allen's test	Normal Prolonge d or incoagul-able
	b) N_2-mustard therapy: 1) man. 2) exp. animals	?	Allen's Test	Prolonged or normal
	c) Aminopterin therapy: 1) man 2) Exp. animals	?	Allen's Test	-do-
	d) Blood dyscrasias: 1) thrombocytopenic purpura 2) Leukemia etc.	?	" "	-do-
3	a) Menorrhagia b) Postpartum hemorrhage	?	Allen's Test	Normal or Prolonged
4	Secondary to vascular occlusion and other conditions: a) Myocardial and pulmonary infarction. b) Thrombosis c) Emboli d) Carcinomatosis e) Fractures etc.	?	Whole plasma and dilute (12.5%) Prothrombin time.	Not Reported
5	Anaphylactic and peptone shock: in dogs	Heparin	Chemical and Jaques' test	Incoagu-lable.

Immunization was produced by repeated transfusions.

In some cases of hemophilia-like disease in women there was the possibility of immunization after pregnancy (136,137,26,143, 144,146). In other cases in men and women the etiology was unknown, and the anticoagulant acted as an antithrombin, antithromboplastin, or antithromboplastinogen. Protamine titration methods (Jaques' method) have been negative in these cases.

Group 2 is the group of hemorrhagic diatheses, which has recently been brought to attention by Allen and his co-workers. In 1947 Allen and Jacobson (2) described a hemorrhagic syndrome in dogs, subjected to total body X-irradiation, which they suggested to be caused by an excess of heparin in the blood. Hitherto these hemorrhagic symptoms had been explained by the thrombocytopenia (158) which accompanied the hemorrhages. As evidence of hyperheparinemia they showed reduction in the prolonged clotting time by addition in vitro of toluidine blue or protamine, and separation from the blood of an anticoagulant material (150), which resembled heparin. There was thus good reason to believe that hyperheparinemia occurred in heavily irradiated dogs. This concept was very attractive (161,162 163,164) and was used to explain a variety of hemorrhagic manifestations. The problem was how to demonstrate the presence of heparin, and, because of the difficulty of chemical isolation, a heparin-protamine titration was used. In the original work on irradiated dogs the method of Jaques was used, but in the later work heparin was added to the blood before the test was carried out (43). In Allen's final test (3,160), described in the previous chapter, the coagulability of heavily heparinized blood was restored by the addition of protamine sulfate in vitro. The amount of protamine needed to restore the coagulability in vitro, after one hour, was rather constant in normals.

It was then found, that in thrombocytopenic purpura (43) and in other conditions (159) (X-irradiation (151), nitrogen mustard therapy (151,152), aminopterin administration (153), certain blood dyscrasias (154) and lately in cases of menorrhagia (155,156) and post partum bleeding (157)) more protamine was required to restore the coagulability to normal under the conditions of the test, or, in other words, that the heparin-protamine equivalent had increased. This, then, was interpreted, first as hyperheparinemia, later as evidence of the presence of heparin-like substances in the blood.

Recent publications in the literature do not confirm the concept of hyperheparinemia in these conditions.

Radiation: Cronkite (166) showed that in vitro addition of toluidine blue to the blood of some of the swine, exposed to atomic radiation, restored their prolonged clotting times to normal. The prolonged clotting time was due to the presence of an anticoagulant, but the clotting time was not prolonged in all animals. The main defects after atomic radiation were thrombocytopenia, vascular damage and bowel ulcerations, not a bleeding tendency. Increased fibrinolysis was probably present. If bleeding occurred, the animals died. He concluded, that hyperheparinemia might be present in some cases, but that there was no actual proof of this, because heparin had not been isolated chemically from the blood.

Holden (167) found hypothromboplastinemia, but no increase in clotting time, in irradiated dogs. In chicks (168) there was only a slight prolongation of the clotting time after internal radiation with P^{32}. In man, (170) slightly prolonged prothrombin times, fibrin times and clotting times have been observed after chronic X-ray exposure.

Recently bacterial invasion from the gut (169) has been called

responsible for blood incoagulability and death after irradiation. Rosenthal (133) showed in rabbits, that toluidine blue in vitro prolonged the clotting times of these animals. These results do not confirm the concept of hyper-heparinemia as a constant phenomenon nor as a cause for the coagulation defect, although in some cases the suggestion is very strong. In blood dyscrasias in man, Best and Limarzi (171), using Allen's titration method, showed a variability of the test in normals, and a significant increase in the protamine-heparin equivalent in only some of the pathological cases (leucemia).

Conley (19,149) investigated several cases of blood dyscrasias with a special technique, using siliconed tubes, and could not demonstrate the presence of even small amounts of heparin in these cases, and in only three cases the presence of a circulating anti-coagulant, which was not heparin.

Park and al. (165) used a heparin-toluidine blue titration method in thrombocytopenic purpura and found no differences from normal blood. Rosenthal (36,37) found no evidence of hyperheparinemia in 8 cases of blood dyscrasias, with the toluidine blue titration method.

Thus, in blood dyscrasias several observations do not confirm the concept of hyperheparinemia as the explanation of the coagulation defect. The increased sensitivity of thrombocytopenic blood to added heparin has been mentioned in chapter II.

Group 3: An increased protamine titration was reported by Allen in menorrhagia, in 80 out of 106 cases during profuse menstruation, (156) accompanied by an increased clotting time in 62 cases. But 16 out of 58 normal women showed an increased protamine titration during or between menses, and an increased clotting time in 27 out of 58 cases.

It seems, therefore that the range of normal variation, which Allen used, was too narrow, and that many of the menorrhagic patients would have fallen into the true normal variation. Barnes (172) reported, that in dicumarolized and heparinized patients no hypermenorrhoea occurred, and that in blood dyscrasias few patients showed menorrhagia, which, when it occurred, could be attributed to pelvic disease.

Group 4: Schilling and DeNatale (145) have demonstrated the occurrence of decreased blood coagulability following vascular occlusion (myocardial infarct, thrombosis and embolism), and in some other conditions by using prothrombin times on 100% and 12.5% diluted plasma. The anticoagulant responsible for the hypocoagulability was not identified, but it did not appear to be heparin. The occurrence of a hypocoagulable state after a hypercoagulable period was also observed in some cases after delivery and operation (174), using a silicone technique. These findings are of interest, because they resemble the phenomenon observed after the intravenous injection of clot accelerating material (173).

Group 5: Waters, Markowitz and Jaques (4,128) demonstrated the presence of hyperheparinemia in dogs after anaphylactic or peptone shock by use of the protamine titration test and by chemical purification. They proved that hyperheparinemia existed in these conditions. Other substances, injected into dogs, which caused (175,176) release of histamine from the dogs' liver, also caused decreased coagulability, probably in part due to hyperheparinemia. It is interesting to note that the dog is a useful animal, in contrast to other species, for the demonstration of hyperheparinemia.

Intravenous therapy with protamine sulfate and toluidine blue:

This has been advocated by Allen (2,43,58,155,156,157) in hemorrhagic conditions due to irradiation, nitrogen mustard therapy, aminopterin administration, menorrhagia and blood dyscrasias. Protamine sulfate or toluidine blue reduced the bleeding tendency, sometimes shortened the clotting time, and restored the increased protamine titration, towards normal. The latter effect, however, cannot be taken as proof of the restoration of the clotting defect to normal, because it may be the effect noted after adding more protamine to the tubes in the protamine-titration test.

Holoubek (177) found occasional effect on bleeding tendency. Park and al. (165), Dreskin (26) and Conley (19) found this therapy of no use in blood dyscrasias. Conley observed a prolongation of the clotting time in two patients with leukemia after intravenous administration of these substances. Allen later reported that in idiopathic thrombocytopenia splenectomy was the treatment of choice.

Protamine sulfate diminished the number of petechiae which developed in experimentally produced thrombocytopenic purpura in animals (117) and this may suggest that it exerts its influence on a capillary defect.

CHAPTER V.

Methods :

1) Preparation of solutions :

The heparin used was obtained from the Connaught Laboratories, Toronto, Canada. This preparation contains approximately 100 mgm. of heparin per 10 cc. The heparin is in the form of the sodium salt. As a preservative 0.3% tricresol is added.

For convenience, 1 mgm. of heparin per 0.1 cc. heparin Connaught was taken as the standard for the investigations.

The protamine sulfate was obtained through the courtesy of the Connaught Laboratories in Toronto, Canada, in the form of a dry powder. It was kept in a desiccator. (Instead of protamine sulfate the word "protamine" is sometimes used.)

Dry materials were weighed on a micro scale.

Fluids were measured with standardized micropipettes.

Solutions were made up in volumetric flasks, and kept at 4° C., when not in use.

Solutions used are mentioned in Table II on next page.

TABLE II

Date of Preparation:	Abbreviation:	Contains:	Strength:
1)	Saline	0.9 grams of sodium chloride U.S.P. per 100 cc. Taken from Baxter vacoliter bottles, labelled "normal saline".	0.9% saline
2)	Hep. Conn.	Heparin Connaught. Contains 100 mgm. heparin in 10 cc. +0.3% tricresol.	1 mgm. heparin in 0.1 cc.
3) August 9/48	H_2	5 cc. Hep. Connaught in 50 cc. saline	100 gamma heparin in 0.1 cc.
4) August 13/48	H_3	25 mgm. Heparin Connaught in 250 cc. saline	10 gamma heparin in 0.1 cc.
5) September/48	H_4	as H_3	-do-
6) made up in small amts. as needed.	R	4 cc. H_4 + 6 cc. saline	4 gamma heparin in 0.1 cc.
7) August 9/48	P_2	100 mgm. protamine sulfate + 0.1 cc. cresolisomers in 100 cc. saline	100 gamma protamine sulfate in 0.1 cc.
8) August 13/48	P_3	25 mgm. protamine sulfate + 0.25 cc. cresolisomers in 250 cc. saline	10 gamma protamine sulfate in 0.1 cc.
9) September 17 1948	P_4	as P_3	-do-
10) June 4/49	P_{A2}	50 mgm. protamine sulfate in 50 cc. distilled water (slight heating increases the solubility of protamine sulfate).	100 gamma protamine sulfate in 0.1 cc.
11) July, 1949	P_{A3}	As P_{A2}	-do-
12) September 16,1949	P_{A4}	-do-	-do-
13) November, 1949	P_{A5}	-do-	-do-
14) June 7,/49	P_{A2} dil.	1 cc P_{A2} + 9 cc. saline	10 gamma protamine sulfate in 0.1 cc.
15) September 23/49	D_1	0.2 cc. T-1824 (= Evans blue) in 100 cc. saline	1 gamma dye in 0.1 cc.

(T-1824 contains 5 mgm. dye in 1 cc. fluid.
0.2 cc. T-1824 contains 1 mgm. dye.
T-1824 used, was Warner's, lot 11.)

2) Cleaning of glassware:

The tubes were cleaned with soap and water and all traces of blood were carefully removed. The tubes were put in bichromate-sulfuric acid for 24 hours, and then were rinsed with hot tap water until the rinsing fluid was colourless. They were then rinsed 10 times with hot tap water and 5 times with distilled water and dried in an oven.

Pipettes were cleaned, put overnight into acid, well rinsed with hot tap water and distilled water, and dried in an oven.

Syringes and needles were rinsed immediately after use, before clotting could take place. They were carefully checked for traces of blood.

3) Siliconing of needles and syringes:

Jaques' method (13,14) was used. Syringes were treated with silicone, rinsed ten times with distilled water to which a few drops of liquid ammonia were added, and then were rinsed three times with hot tap water and three times with distilled water. They were dried in the oven and autoclaved.

Needles were treated with silicone, then with the dilute ammonia water, with hot tap water, distilled water, dried in the oven and autoclaved.

4) Clotting tests:

A) Technique of protamine-heparin titration (Jaques' method).

a) Estimation of the equivalent between protamine and heparin:

The blood was obtained from donors at the blood bank. After removal of the needle from the vacuum bottle, 5 to 10 cc. of blood

was collected in a free flow in a large testtube, and with a 5 cc. pipette portions of 0.5 cc. of blood were placed from right to left into a series of testtubes of 11 mm. internal diameter, containing increasing amounts of protamine sulfate and a standard amount of heparin in 0.5 cc. of saline. The protamine and heparin solutions were measured from calibrated burettes and the saline was pipetted into the tubes.

The stopwatch was started as soon as the blood was added to the tubes, which were then corked. The contents of the tubes were well mixed, but care was taken that the blood did not touch the cork. The tubes were then placed into a waterbath at 37°C. and were tilted at 30 second intervals until the blood was clotted. It was considered the endpoint when the tube could be inverted 180° without the blood spilling. In cases in which there was a marked heparin excess this criterion could not always be maintained, as only partial clot formation took place in some cases. This was mentioned in the reports of the clotting tests.

In some instances the blood was obtained by venapuncture with a 20 gauge needle and a 10 cc. syringe. After removal of the needle the blood was put into a large testtube and the remainder of the test was performed as above.

Any modifications of this technique are mentioned in the reports. b) The same technique, without in vitro addition of heparin was used to determine heparin level in a given blood sample.

B) <u>Technique of the heparin-protamine test of Allen</u>:

a) Blood was obtained by clean venapuncture, with slight stasis, with a siliconed 20 gauge needle and 2 siliconed syringes of 10 and 20 cc. Blood from the second siliconed syringe was put into a clean large testtube, marked at the 10.1 cc. level and containing

0.1 cc (= 1 mgm.) of Heparin Connaught. The tube was stoppered and the contents mixed by inverting the tube several times. As soon as possible, but always within 45 minutes, the following test was performed. The tube was inverted 10 times to mix the contents, and with a 5 cc. pipette one cc. of heparinized blood was measured into each of 6 test tubes of 11 mm. internal diameter, containing 0.10, 0.12, 0.14, 0.16, 0.18 and 0.20 cc. of a protamine solution ($P_{A\ 1-5}$). These test tubes were covered with waxed paper, inverted 10 times, corked and left at room temperature. They were inspected for clotting after 60 minutes, 120 minutes and 24 hours. The stopwatch was started as soon as the blood was put into the tubes. The tubes were filled from left to right. (Each tube contained 1 cc. blood plus 99 gamma of heparin (1000 gamma/10.1 = 99 gamma) and increasing amounts of protamine, ranging from 100 to 200 gamma in 0.1 cc. of distilled water. For the sake of convenience each tube was considered to contain 100 gamma of heparin).

Variations in the technique:

b) In some tests the tubes were inspected for coagulation after 15, 30, 45, 60, 120 minutes and 24 hours.

c) In a number of instances a second test was performed and the tubes with the heparin-protamine blood mixture were placed in a waterbath at 37°C. These tubes were tilted every 30 seconds until total clot formation took place. If only partial clot formation took place this was indicated by the letters P.C.

d) In a number of cases a third test was performed. The venous blood was transferred from a siliconed syringe into a large test tube (containing 0.2 cc. (= 2 mg.) of heparin Connaught) to the 10.1 cc. level. After mixing, 0.5 cc. of the heparinized blood was pipetted into each of a series of small test tubes of 11 mm.

internal diameter, containing 0.10 to 0.20 cc. of a protamine solution (P_{A1} to P_{A5}) to which normal saline was added up to 0.5 cc. The tubes were covered with waxed paper, inverted ten times to obtain proper mixing, corked, put in a waterbath at 37°C and investigated for clotting at 30 second intervals.
(Each of these tubes contained 0.5 cc. blood plus 99 gamma of heparin and 100 to 200 gamma of protamine in 0.5 cc. saline. For the sake of convenience the amount of heparin in each tube was considered to be 100 gamma.)

C) Technique of the protamine test:

A clean venapuncture was performed (with slight stasis), using a siliconed 20 gauge needle and a siliconed 10 cc. syringe. As soon as the blood appeared in the syringe the tourniquet was released, 5 cc. of blood was drawn, after which the syringe was changed for a second siliconed 10 cc. syringe, into which 8 cc. of blood was drawn. These blood samples were used for other coagulation tests. Then a third, non-coated, 20 cc. glass syringe was attached to the needle and another 20 cc. of blood was drawn, which was used for several tests. 10 cc. of the blood in the third syringe was poured into a large test tube and with a 5 cc. pipette 0.5 cc. portions were rapidly transferred into a series of test tubes of 11 mm. internal diameter (containing increasing amounts of protamine sulfate in saline (see below), and 1 cc. of blood was put into an empty 11 mm. test tube. The stopwatch was started as soon as the blood appeared in the first siliconed syringe. The contents of the tubes were well mixed, but care was taken that the blood did not touch the corks, with which the tubes were stoppered. The tubes were then placed in a waterbath at 37°C. and were tilted, after 6 minutes, at 30 second intervals until the blood was clotted.

It was considered the endpoint when the tube could be inverted without the blood spilling. In the tubes containing large amounts of protamine, in which the clotting time was markedly prolonged under pathological circumstances (see tests), only partial clot formation took place in some cases. This was mentioned in the reports of the tests. The tubes were filled from right to left. The protamine sulfate solution and the saline were measured in the tubes with standardized, calibrated 1 cc. pipettes.

The tubes contained:

Protamine sulfate cc. :	0	0	2	4	6	8	10	12	16
Saline cc.	-	0.50	0.48	0.46	0.44	0.42	0.40	0.38	0.34
Blood cc. :	1.-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

D) <u>One Stage Prothrombin time determination</u>:

4.5cc of the blood in the first syringe was put into a centrifuge tube containing 0.5 cc. of a 0.1 Molar sodium oxalate solution. The oxalated blood was used for the <u>prothrombin time</u> determination. The blood was centrifuged at 1,500 revolutions per minute for five minutes and the plasma removed. Two serologic tubes, each containing 0.1 cc. of the stored frozen thromboplastin extract (Difco), were warmed in a water bath at 37.5°C. for ten minutes. 0.1 cc. of the plasma to be tested was added and the tubes were returned to the water bath for thirty to sixty seconds. Exactly 0.1 cc. of an 0.025 Molar calcium chloride solution was drawn into a tuberculin syringe through a 20-gauge hypodermic needle. The solution was squirted from the syringe into the thromboplastin-plasma mixture, and a stopwatch was started simultaneously. A wooden applicator was plunged rhythmically into the mixture and

observed through the wall of the waterbath to note the moment of adhesion of the clot to the applicator. When the first clot appeared, the watch was stopped. All determinations were done in duplicate, and closely agreeing values were averaged.

E) Control clotting time :

One cc. of the blood, obtained from the second syringe, was put into each of two test tubes of 11 mm. internal diameter. The tubes were placed in a waterbath at 37°C and, after 6 minutes, were tilted every 2 minutes until the tube could be inverted without spilling the blood. The longest clotting time was taken as the control clotting time. Normal range : 20-26 minutes.

F) Heparin clotting time :

Blood from the second syringe was put into each of two clean test tubes of 11 mm. internal diameter to the 1.1 cc. mark. Each tube already contained 4 gamma of heparin in 0.1 cc. saline. The tubes were placed in the waterbath at 37°C. and after 15 minutes were tilted every minute until clot formation took place.

G) Bedside technique for prothrombin time determination :

0.1 cc. of rabbit brain extract was placed in a watch glass. One cc. of venous blood was added, and simultaneously the stop-watch was started. The blood and the extract were mixed with a needle, which was then rhythmically lifted from the blood. As soon as a thread was visible the watch was stopped, and this was taken as the prothrombin time. (normal values : 18 to 20 seconds).

CHAPTER VI

Experimental Work.

Experiment number 1:

Objective of experiment: to determine the heparin-protamine equivalent between H_2 and P_2.

Technique: Jaques' method of heparin-protamine titration was used, as described at page 34, with the exception that this test was performed at room temperature.

Solutions: H_2, containing 100 gamma of heparin in 0.1 cc. of saline, and P_2, containing 100 gamma of protamine in 0.1 cc. of saline.

Results: see Table III, and figure 1 A.

Comment: Each curve shows a V-shape, with a sharp angle between the two limbs. With increasing amounts of protamine in the tubes, the clotting time decreases until it reaches a minimum at the angle of the curve, and then increases again (right limb of the curve). This is interpreted in such a way that at the angle of the curve, where the clotting time is shortest and equals the clotting time in the control tube (without heparin or protamine), heparin or protamine exerts no influence on the clotting time. At that point, the anticoagulant effect of heparin is completely neutralized by protamine. In the tubes, containing smaller amounts of protamine, the clotting time is prolonged due to the anticoagulant effect of heparin, and inthe tubes containing larger amounts of protamine, the clotting time is prolonged due to the anticoagulant effect of protamine, which is a much weaker anticoagulant than heparin. The point of the angle of the curve is called the equivalence point, because the protamine effect equals the heparin effect on the clotting time.

Thus, in this test, 140 gamma of protamine equals 100 gamma of

heparin. The <u>ratio</u>, or <u>equivalent</u>, between heparin and protamine is 100/140. The ratio is <u>constant</u> in this test.

Thus : Heparin/protamine= 100/140.

or $H_2/P_2 = 100/140$

TABLE III

Each tube contains 100 gamma heparin + 0.5 cc. saline + 0.5 cc. blood, plus :

Protamine gamma/tube :	120	140	160	180	200	Control. 0.5cc. Saline + 0.5 cc. blood.
DONORS :	CLOTTING TIMES (min) at R.T.					
A	> 30	12	13½	16	17	10½
B	> 25	9	10	10½	12	7
C	> 25	9	14	13	21	11
D	> 19	5¾	8	8	11½	4½
E	> 20	10¼	13	13	14½	10
F	> 20	15	18½	22	18½	14½

Experiment number 2 :

Objective of experiment : to determine the heparin-protamine equivalent between H_3 and P_3.

Technique : is described on page 34. (Jaques' method).

Solutions : H_3, containing 10 gamma of heparin in 0.1 cc. saline, and P_3, containing 10 gamma of protamine in 0.1 cc. saline.

Results : are shown in Table IV and figure 1B.

Comment : In this test much weaker heparin and protamine solutions are used than in the first experiment. The clotting times are determined in a constant waterbath at 37°C. The heparin-protamine equivalent under the circumstances of the experiment is not constant in the seven tests performed. In one instance the equivalent is 10/10, in two instances it is 10/14 and in the remaining 4 tests it is 10/12. The variation is probably due to technical errors (see experiment 3). To find the heparin-protamine equivalent for two given solutions, it is necessary to perform a series of heparin-protamine equivalence tests. The equivalent between H_3 and P_3 is taken as 10/12, because this result is obtained in the majority of the tests.

TABLE IV

Each tube contains 10 gamma heparin + 0.5 cc. saline + 0.5 cc. blood, plus:							Control.
Protamine gamma/tube	8	10	12	14	16	18	0.5 cc. saline + 0.5 cc. blood.
DONORS:	CLOTTING TIME (min) at 37°C.						
A	16½	9½	6½	5½	7	7½	7
B	22	5½	4	4	4¼	4¼	4¾
C	19	11½	6	8½	7½	9	6½
D	13	4½	3	3½	4½	3½	3½
E	15	5½	6	6	6	-	6
F	10	6¾	3½	4	4	4	4
G	8½	5½	3½	3	3¾	4¼	3

Experiment number 3

Objective of experiment: to determine the range of variation in the determination of clotting times in a series of tubes containing equal amounts of saline and blood (Table V a), and equal amounts of a heparin-protamine mixture in saline and blood (Table V b).

Technique: is described on page 34. (Jaques' method for heparin-protamine titration.)

Solutions: H_3, containing 10 gamma of heparin in 0.1 cc. of saline, and P_3, containing 10 gamma of protamine in 0.1 cc. of saline.

Results: are shown in Tables Va and Vb.

Comment: In Table V a the greatest difference between minimum and maximum clotting time in a series of 5 tubes is 1½ minutes, and in table V b the greatest difference is 2¼ minutes. This series is too small for statistical analysis. It can be seen, that there is a considerable degree of variation between serial estimations of clotting times under standard conditions, due to technical errors (measuring of fluids, mixing, estimation of endpoints, etc.). The range is greater when heparin-protamine solutions are used, (table V b) than when saline is used (table V a), supposedly due to the fact that the chances of making errors in measurement of heparin and protamine solutions are more numerous.

In the determination of the equivalence point in protamine-heparin titrations the above demonstrated variability has to be taken into account.

TABLE V a

Each tube contains 0.5 cc. saline + 0.5 cc. blood.

Tubes :	1	2	3	4	5	
DONORS :	CLOTTING TIMES (Min.) at 37°C.					RANGE (min) in tubes 1 to 5
A	7½	8¼	8½	7	8	1
B	4¼	4	4	4	4	¼
C	3½	4¼	4	4	3½	¾
D	5	4½	6	5	6	1½
E	6	5	6	6	6	1

TABLE V b

Tube :	1	2	3	4	5	6	
Each tube contains 10 gamma heparin + 12 gamma protamine in 0.5 cc. saline + 0.5 cc. blood.						contains 0.5 cc. saline + 0.5 cc. blood.	
DONORS :	CLOTTING TIMES (min.) at 37°C.						Range (min.) in tubes 1 to 5
A	5¼	5¼	6¼	7½	5¾	5¾	2¼
B	5¼	6¾	5½	7¼	7¼	4¼	2
C	5¼	6¼	5½	5½	4¼	6¼	2
D	4¼	4¾	6	6½	5½	5	2¼
E	6¾	6	6½	6	6	5¾	¾

Experiment number 4 :

Objective of experiment : to determine the heparin-protamine equivalent between H_3 and P_3.

Technique used : as described on page 34 (Jaques' method).

Solutions used : H_3, containing 10 gamma of heparin in 0.1 cc. of saline, and P_3, containing 10 gamma of protamine in 0.1 cc. of saline.

Results : are shown in Table VI and in figure 1C.

Comment : In this experiment the difference between the amounts of protamine in the tubes in greater than in experiment number 2. As a result the difference between the clotting times in the different tubes increases. The angle of the curve becomes sharp, and the equivalent between heparin and protamine is easier to read. It is in 4 out of 5 instances 10/12. The heparin-protamine ratio can therefore be assumed to be 10/12. Thus, when the difference between the amounts of protamine in the tubes is greater, the heparin-protamine ratio is easier to read, due to the formation of a sharp angle in the curve. The conclusion cannot be drawn that the ratio under those circumstances is more sharply defined, as it is uncertain from this test, whether it is 10/10, 10/12 or 10/14.

TABLE VI

Each tube contains 10 gamma of heparin + 0.5 cc. saline + 0.5 cc. blood, plus:				Control
Protamine gamma/tube	8	12	20	0.5 cc. saline + 0.5 cc. blood
DONORS:	C.T. (min.) at 37°C.			
A	17½	4½	6¼	4¼
B	18½	3¾	4½	5¼
C	12	5¼	5¼	4½
D	18	7	6	5
L	22	4¼	4¼	4¾

Experiment number 5:

Objective of experiment: to determine the heparin-protamine ratio between H_3 and P_3 in duplicate estimations on the same blood samples.

Technique: as described on page 34. (Jaques' method).

Solutions: H_3, containing 10 gamma of heparin in 0.1 cc. of saline and P_3, containing 10 gamma of protamine in 0.1 cc. of saline.

Results: are shown in Table VII.

Comment: Duplicate estimations in this test give rather satisfactory results, with this restriction, that when heparin is markedly in excess, duplicate estimations do not show close agreement.

The H_3/P_3 ratio is again 10/12.

TABLE VII

Each tube contains 10 gamma of heparin + 0.5 cc. saline + 0.5 cc. blood, plus:					Control.
Protamine Gamma/tube	5	8	12	20	0.5 cc. saline + 0.5 cc. blood
DONORS:	CLOTTING TIMES (min) at 37°C.				
A	33	19	4	5	4
	>48	19	$4\frac{1}{2}$	5	-
B	>33	17	$3\frac{3}{4}$	$4\frac{1}{2}$	4
	>33	>33	4	$5\frac{1}{2}$	-
C	21	$10\frac{1}{2}$	$4\frac{1}{4}$	$4\frac{3}{4}$	4
	>27	$10\frac{1}{2}$	$4\frac{1}{2}$	$7\frac{1}{4}$	-

Experiment number 6:

Objective of experiment: to compare the heparin-protamine equivalent, using different amounts of heparin in each tube.

Technique: as described on page 34. (Jaques' method).

Solutions: H_3, containing 10 gamma of heparin in 0.1 cc. of saline, and P_3 and P_4, each containing 10 gamma of protamine in 0.1 cc. of saline.

Results: see Table VIII and figure 2.

Comment: The equivalent between H_3 and P_4 seems to be 2/3. The equivalents between H_3 and P_3 seem to be 5/6, 10/12 and 20/24. In other words the heparin-protamine ratio, under constant conditions, is constant. 2 gamma of heparin added to 0.5 cc. blood can be demonstrated in this way.

TABLE VIII

Each tube contains 2 gamma heparin +0.5 cc. saline + 0.5 cc. blood, plus					Control: 0.5 cc. saline + 0.5 cc. blood	(H_3 and P_4 used.)
Protamine gamma/tube:	1	2	3	4		
DONOR:	CLOTTING TIME (Min) at 37°C.					
A	6½	5½	3½	4	3½	
B	13½	12	7	7	7	
C	11½	9½	8	7½	8	

Each tube contains 5 gamma heparin +0.5 cc. saline +0.5 cc. blood, plus:				Control: 0.5cc saline + 0.5 cc. blood	(H_3 and P_3 used.)
Protamine gamma/tube.	4	6	8		
DONOR:	CLOTTING TIME (Min) at 37°C.				
D	>15	6	8	6	
E	13	8¼	5¾	5¾	
F	10	4¾	6½	7½	

Each tube contains 10 gamma heparin + 0.5 cc. saline + 0.5 cc. blood, plus:				Control: 0.5 cc. saline + 0.5 cc. blood.	(H_3 and P_3 used.)
Protamine gamma/tube	8	12	20		
DONOR:	CLOTTING TIME (Min) at 37°C.				
G	17½	4½	6¼	4¼	
H	18½	3¾	4½	5¼	
K	12	5¼	5¼	4½	

Table VIII cont'd.

Each tube contains 20 gamma heparin + 0.5 cc. saline + 0.5 cc. blood, plus					Control (H_3 and P_3 used.)
Protamine gamma/tube:	20	24	28	32	0.5 cc. saline + 0.5 cc. blood.
DONOR:	CLOTTING TIME (Min). at 37°C.				
L	13½	8	8	-	6
M	14	6½	8	8½	6¼
N	11½	5¼	6½	5¾	5¾

Experiment number 7

Objective of experiment: establishment and comparison of heparin-protamine equivalents, using different solutions of heparin and protamine.

Technique: as described on page 34 (Jaques' method). One test was carried out with siliconed needles, syringes and pipettes and the clotting times were estimated in siliconed test tubes.

Solutions: H_3 and H_4, each containing 10 gamma of heparin in 0.1 cc. of saline, and P_3, P_4, P_{A2} dil. and P_6, each containing 10 gamma of protamine in 0.1 cc. of saline.

Results: see Table IX.

Comment: H_3/P_3 = 10/12 in most of the tests.

H_3/P_4 = 10/12. The use of silicone technique, in one instance, does not alter the equivalent. The difference between the clotting times in the tubes is greater, when silicone technique is used.

H_4/P_4 = 10/12.

H_4/P_{A2} dil. = 10/12.

H_4/P_6 = 10/12 in 3 instances, and = 10/14 in 2 instances. Here the equivalent is probably somewhere between 12 and 14 gamma protamine for every 10 gamma of heparin.

In general it may be said that the heparin-protamine equivalent under the conditions of the test is rather constant with the use of different solutions of the same strength. There is no difference observed in heparin-protamine equivalent between blood specimens taken from males or females in these experiments.

(M) = Male

(F) = Female.

TABLE IX

Each tube contains 10 gamma heparin + 0.5 cc. saline + 0.5 cc. blood, plus:							Control.	
Protamine gamma/tube:	8	10	12	14	16	18	0.5 cc. saline + 0.5 cc. blood	Solutions and H/P ratios:
DONORS:	CLOTTING TIMES (Min) at 37°C.							
A	16½	9½	6½	5½	7	7½	7	H_3/P_3 =
B	22	5½	4	4	4¼	4¼	4¾	10/12
C	19	11½	6	8½	7½	9	6½	
D	13	4½	3	3½	4½	3½	3½	
E	15	5½	6	6	6	-	6	
F	10	6¾	3½	4	4	4	4	
G	8½	5½	3½	3	3¾	4¼	3	
Venapuncture blood:								
H	-	4¾	2½	2½	2½	-	3¼	H_3 / P_4 =
K	-	12	6½	6½	8½	-	6½	10/12
B.F. (F)	48	17	12	15	15	-	13	H_4 / P_4 =
B.T. (M) (Sil. tubes)	>90	46	36	46	44	-	28	10/ 2
Mrs. M. (F)	29	10½	10	11½	11½	-	11	H_4/P_{A2} dil. =
Mrs. S. (F)	30	13	12	12	16	-	11	10/12
Dr. R. (M)	24½	10½	9½	12	11½	-	9½	
X (F)	17	9½	6½	6½	8½	10½	6½	H_4/P_6 =
F.S. (F)	>20	16½	13	15	14½	18½	13	10/12 (3x)
N.L. (F)	>30	19½	16½	18	18	18½	14	10/14 (2x)
M.H. (F)	>21	14½	15	14	15	15	-	
N. (F)	18	13½	14	13	14	15	11½	

Experiment number 8 :

Objective of experiment : to investigate the influence of the dye T-1824 on the heparin-protamine equivalent.

Technique : as described on page 34 (Jaques' method).

Solutions : H_3, containing 10 gamma of heparin in 0.1 cc. of saline, P_4, containing 10 gamma of protamine in 0.1 cc. of saline, and D_1, containing 1 gamma of dye (T-1824) in 0.1 cc. of saline.

Results : see tables X a and b, and figure 1 D.

Comment : The heparin-protamine equivalent for H_3 and P_4 is 10/12. (table X a). After addition of the dye solution the equivalent has changed to 10/14. (table X b, and figure 1 D).

Addition of 0.1 cc. of D_1 has little influence on the clotting time, compared with a control tube (table X b).

TABLE X a

Each tube contains 10 gamma heparin + 0.5 cc. saline + 0.5 cc. blood, plus:						Control.
Protamine gamma/tube:	8	10	12	14	16	0.5 cc. saline + 0.5 cc. blood.
DONORS:	CLOTTING TIMES (Min) at 37°C.					
A	14½	7¾	5½	5½	5½	5½
B	10¾	7¾	5½	6¼	5½	4½
C(venap.)	11	8	5½	5½	6	5½

TABLE X b

Each tube contains 10 gamma heparin + 0.4 cc. saline + 0.1 cc. D_1 + 0.5 cc. blood, plus:						Control	Control + dye
Protamine gamma/tube	8	10	12	14	16	0.5 cc. saline + 0.5 cc. blood	0.4 cc. saline + 0.5 cc. blood + 0.1 c c. D_1
DONORS:	CLOTTING TIMES (Min.) at 37°C.						
D	>22	9½	8½	3	3½	3½	3½
E	25	13	8	5½	5½	4	5½
C (Venap.)	>25	11	7	5	6½	4½	5½

Experiment number 9 :

Objective of experiment : to investigate the heparin-protamine equivalent in recalcified dextrose-citrate blood.

Technique : as described on page 34 (Jaques' method). The dextrose-citrate blood was measured with a 5 cc. pipette in the tubes containing the heparin-protamine-calcium chloride mixtures and the clotting times were determined in the usual manner.

Solutions : H_3, containing 10 gamma of heparin in 0.1 cc. of saline, P_4, containing 10 gamma of protamine in 0.1 cc. of saline, 1% $CaCl_2$ solution, and dextrose-citrate blood from Baxter vacoliters. (Baxter vacoliters contain 2.3 grams of dextrose, and 1.7 grams of sodium citrate per 100 cc., buffered with a sodium phosphate mixture, according to the McGill formula).

Results : see tables XI a and b, and figure 1 D.

Comment : The heparin-protamine equivalent in venous blood, to which 0.1 cc. of a 1% $CaCl_2$ solution is added in each tube, is 10/12. The $CaCl_2$ solution in itself seems to have no influence on the heparin-protamine equivalent, nor on the clotting time in this experiment. In table XI b it is shown that the heparin-protamine equivalent in recalcified dextrose-citrate blood is 10/10. Thus the dextrose-citrate mixture causes a shift of the equivalent.

TABLE XI a

Each tube contains 10 gamma heparin + 0.4 cc. saline + 0.1 cc. $CaCl_2$ 1% + 0.5 cc blood, plus :					Control	Control + $CaCl_2$
Protamine gamma/tube :	8	10	12	16	0.5 cc. saline + 0.5 cc. blood.	0.4 cc. saline + 0.1 cc. $CaCl_2$ 1% + 0.5 cc blood.
DONORS :	CLOTTING TIMES (Min) at 37°C.					
A	13	10½	6½	7½	6	6½
B	18	12½	7	7½	5½	5½

TABLE XI b

Each tube contains 10 gamma heparin + 0.4 cc. saline + 0.1 cc $CaCl_2$ 1% + 0.5 cc. dextrose-citrate blood, plus :					Control
Protamine gamma/tube :	8	10	12	16	0.4 cc. saline + 0.1 cc. $CaCl_2$ 1% + 0.5 cc. dextrose-citrate blood
CLOTTING TIMES (min) at 37°C.					
Bottle 4793	10	6	6	5½	5
Bottle 4793	9	5½	6	5½	5

Experiment Number 10:

Objective of experiment: to estimate the heparin levels in the blood by the heparin-protamine titration method of Jaques after intravenous injection of 20 mgm. of heparin Connaught.

Technique: see on page 35, (Jaques' method). No heparin was added to the tubes.

Solution: P_4, containing 10 gamma of protamine in 0.1 cc. of saline.

Results: see Table XII and figure 3A.

Comment: 1) The whole blood clotting times and the clotting times in the tubes containing 0.5 cc. of saline and 0.5 cc. of venous blood are prolonged after the heparin injection, but not much. The whole blood clotting time is shortened by the dilution with saline.

2) The protamine titration test before the injection shows a slight anticoagulant effect of protamine on the clotting time. In the protamine titration tests after the heparin injection the control clotting times are prolonged and there is an angle in the curve, thus demonstrating the neutralizing effect of protamine on the heparin present in the blood. This angle is best demonstrated in the titration curve 33 minutes after the heparin injection, the equivalent being $H/P_4 = x/2$. Thus H= 1.66 gamma of heparin per 0.5 cc. of blood, or 3.3 gamma per cc. of blood. The amount of heparin present in the blood, 33 minutes after intravenous injection of 20 mgm. heparin Connaught, is therefore approximately 3.3 gamma, as calculated by the heparin-protamine titration test. The protamine titration tests carried out 15 and 60 minutes after the injection also show a slight angle, but the differences in clotting times are too small to be convincing evidence of protamine effect.

A rough calculation of the amount of heparin present in the blood after the injection of 20 mgm. in a normal size man gives 20 mgm. distributed in a blood volume of 5000 cc. which is a concentration of 4 gamma of heparin per cc. of blood. The heparin level found by the titration method is in rather good agreement with the calculated maximal concentration in the blood.

TABLE XII

Each tube contains 0.5 cc. saline + 0.5 cc. blood, plus:								Control	Control
Protamine gamma/tube	2	4	6	8	10	12	16	0.5 cc. saline + 0.5 cc. blood	1 cc. Blood.
Sample:	CLOTTING TIMES (Min) at 37°C.								
Before inj.	10½	10½	11	12	12	11½	12½	8	12
15 min. after inj. 20 mgm. heparin	9	10½	10½	11¾	13	11½	14	10½	14
33 min. after inj.	8½	10	10½	11	11	13½	12	12	12½
60 min. after inj.	9	10	11	12	14	14	14	10	11

Experiment number 11:

Objective of experiment: to determine the heparin levels in the blood by the heparin-protamine titration method of Jaques after the intravenous injection of 50 m.gm. of heparin Connaught.

Technique: as described on page 35,b. No heparin was added to the tubes.

Solution: P_4, containing 10 gamma of protamine in 0.1 cc. of saline.

Results: are shown in table XIII, and figure 3B.

Comment: 1) The titration curve before the heparin injection shows an irregularity due to a technical error.

2) After the heparin injection the titration curves show a completely different character. The control clotting times are markedly prolonged, and in the tubes containing protamine there is partial or total suppression of the heparin effect. It is however difficult to estimate the heparin-protamine equivalent accurately, because in none of the curves is there a sharp angle present. For instance, in the curve 10 minutes after the injection it is hard to decide whether to take the equivalent at 10 gamma or at 6 gamma of protamine. In the curve taken 30 minutes after the injection it seems feasible to take the equivalent at 8 gamma of protamine.

Calculated heparin levels in the blood are then as follows: 10 minutes after injection: $H/P_4 = x/10$. H = 8.3 gamma in 0.5 cc. of blood. Heparin level is 16.6 gamma per cc. Or, if $H/P_4 = x/6$ is taken, the heparin level would be 10 gamma per cc.

30 minutes after injection: $H/P_4 = x/8$. H = 6.6 gamma per 0.5 cc. of blood. The heparin level would be 13.2 gamma per cc. of blood.

60 and 135 minutes after the injection the differences in the clotting times are still less pronounced and it becomes even more speculative to calculate heparin levels by this method. The control

clotting times however are still markedly prolonged due to the heparin effect. A rough calculation from the administered dose of heparin and blood volume shows that if 50 mgm. of heparin is distributed in approximately 5000 cc. of blood, the heparin level can reach a maximum value of 10 gamma per cc. blood. This would implicate that the heparin level of 16.6 gamma per cc., calculated from the titration method, is much too high and has to be discarded. It is therefore feasible to take the heparin level 10 minutes after the injection as 10 gamma per cc. Consequently the calculated level of 13.2 gamma of heparin 30 minutes after the injection is too high.

In conclusion it can be said, that, using the heparin-protamine titration method, one is able to demonstrate a neutralizing effect of the heparin by the protamine, that it is difficult and in some instances impossible to calculate accurate heparin levels by the method used, and that the smallest amount shown was 3.3 gamma of heparin per cc. Smaller amounts were not demonstrated and it is highly improbable, that they can be demonstrated by this method. These conclusions concern the demonstration of intravenously administered commercial heparin.

Irregularities in the titration curves in this and in the former experiment may be due to the fact, that the blood now contains the anticoagulant. The pipetting of blood has to be done rather fast, with a 5 cc. pipette, so that errors in pipetting the blood influence the outcome of the test.

TABLE XIII

	Each tube contains 0.5 cc. saline + 0.5 cc. blood, plus:							Control	Control
Protamine gamma/tube:	2	4	6	8	10	12	16	0.5 cc. saline + 0.5 cc. blood	1 cc. blood
Sample:	CLOTTING TIMES (Min.) at 37°C.								
Before inj.	11	11	11	15½	13½	12½	13½	9½	8
10 min. after inj.	25	13¼	10½	10½	9	10¼	9¾	47 P.C.	62 P.C.
30 min. after inj.	17	12½	11½	8¾	9½	10½	10½	33	52 P.C.
60 min. after inj.	12½	10½	9½	9½	9½	13½	10	32	36
135 min. after injection	11¼	11¼	14	14	12	14	12	12	16½

P.C. = Partial Clot

Experiment number 12 :

Objective of experiment : to establish normal values for the anti-coagulant effect of protamine sulfate on blood coagulation in vitro.

Technique : is described on page 37 (Protamine test).

Solution : P_4, containing 10 gamma of protamine in 0.1 cc. of saline.

Results : shown in tables XIV and XV, and in figure 4.

Comment : from the figure it can be seen that 1) protamine sulfate under the conditions of the test exerts an anticoagulant effect on the in vitro clotting time. The effect increases with increasing amounts of the material. However, the curves flatten at the end, which may be explained by technical errors. (The blood in the tubes, containing the largest amount of protamine, is the blood that leaves the pipette first. It is often observed in this test, and in other coagulation tests, that when blood is measured with a pipette or a syringe, the clotting time in the tube, which receives the first sample of blood from the pipette or from the syringe, shows an increased rate of coagulation).

2) There is some shortening of the clotting time in the tube containing equal amounts of saline and blood, compared with the clotting time in the tube containing whole blood. This is observed in 12 out of 20 cases. The difference is not significant. (It is less than 3 times the standard error of the difference between the two means). (178).

The range and mean values of the clotting times in the tubes, containing increasing amounts of protamine sulfate, are determined. The difference between the whole blood clotting time for blood taken from males and females is not significant. (it is less than 3 times the standard error of the difference between the 2 means.)

3) Individual curves sometimes show irregularities, due to technical errors in the procedure of the test.

4) It can be seen from the scatter, that the range of clotting times is much wider in the tubes containing greater amounts of protamine sulfate than in those which contain only small amounts of protamine sulfate. The lower limit of the range increases very little with increasing amounts of protamine sulfate. This may be explained by the fact that any shortening influence on the clotting time becomes more apparent in "prolonged" clotting time methods.

The standard error of the difference between the means of whole blood clotting times for male and female blood is 0.73 minutes. The standard error of the difference between the means of the clotting times in whole blood and in a mixture of equal amounts of saline and blood is 0.41 minutes.

TABLE XIV

Protamine test :

Protamine (gamma) :	-	-	2	4	6	8	10	12	16
Saline cc. :	-	0.50	0.48	0.46	0.44	0.42	0.40	0.38	0.34
Blood cc. :	1.00	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
NORMALS :	CLOTTING TIMES (Min.) et 37°C. :								
1	12	12	14½	14½	16½	20½	16½	19½	20½
2	12½	11	11½	14	18½	18½	22	20½	28
FEMALES 3	13	13	14	16	18	23	24½	26½	19½
4	10½	7½	9½	12	12	12½	12½	16	18
5	13	13	13½	14½	15½	17½	17½	22½	20½
6	15	13½	16	16	19½	21	20½	20½	27½
7	10½	12	12½	14½	17½	19	19	19	17½
8	13½	11	11½	12½	15	14	21½	15	22½
9	13½	14	14½	14½	17½	24	28½	31	31
10	14½	10½	11	13	16	20	17½	18	20
11	12	12½	13½	14½	15½	20	20	20	22
MALES 12	12	12½	12½	15	17	23	23½	24½	24½
13	15	14	16	17	20½	21	23½	24½	25½
14	9½	10½	11½	13	14½	16½	18	18	19
15	12	10	10½	12½	14	16	18½	19½	22
16	13	11	11	11½	15½	16½	18½	16½	18½
17	13	12	15½	13½	17½	20	26	22½	21
18	8½	9	10	11	12½	11½	14	14	15
19	11½	10	14	13	14	17	20	19	17
20	13	11½	13	14	17½	16	23½	16	23½

TABLE XV

Protamine test.

Protamine gamma :	-	-	2	4	6	8	10	12	16
Saline cc. :	-	0.50	0.48	0.46	0.44	0.42	0.40	0.38	0.34
Blood cc. :	1.00	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
20 Cases	CLOTTING TIMES (Min) at 37°C.								
Range :	8½-15	7½-14	9½-16	11-17	12-20½	11½-24	12½-28½	14-26½	15-28
Means : Males :	12.25	11.45	12.65	13.53	16.03	18.32	20.86	19.80	21.36
St.Dev.	1.79								
Females :	12.67	11.67	13.17	14.50	16.67	18.67	18.50	20.90	24.00
St.Dev.	1.38								
Males & Females :	12.46	11.56	12.91	14.02	16.35	18.50	20.27	20.15	21.65
St.Dev.	1.68	St. Dev. 1.58							

St.Dev. = standard deviation.

Experiment number 13:

Objective of experiment: to obtain normal values for the clotting time prolonged by the addition of 4 gamma of heparin.

Technique: described on page 39 F.

Solution: R-solution was used, containing 4 gamma of heparin in 0.1 cc. of saline.

Results: see table XVI and figure 5.

Comment: The normal range of the clotting times in this test is rather wide, between 29 and 56 minutes. The mean values for men and women, and the general mean are calculated. The curve is skewed, probably due to the effect of technical errors, which, as explained before, have a tendency to shorten the clotting time.

The endpoint in this test is difficult to determine, due to the formation of a fibrin sheet at the air blood interface. A small air bubble sometimes appears under the upper layer of clotted blood. The endpoint is taken, when this air bubble no longer moves on tiltin g the tube.

TABLE XVI

Heparin (gamma)	4	4	4	4
Saline cc. :	0.10	0.10	0.10	0.10
Blood cc. :	1.00	1.00	1.00	1.00

CLOTTING TIME (Min.) at 37°C.

FEMALES:			MALES:		
1	40	38	1	30	27
2	38	36	2	44	40
3	31	31	3	39	43
4	35	33	4	36	34
5	33	36	5	48	42
6	43	-	6	37	36
7	32	29	7	40	39
8	33	36	8	51	-
9	34	34	9	34	32
			10	46	40
			11	39	-
			12	32	32
			13	36	-
			14	35	40
			15	30	35
			16	32	31
			17	29	32
			18	56	52
			19	32	41
			20	56	49
			21	36	39

Normal Range: 29 - 56

Mean :	F	M	General
	35.1	37.5	36.9

Experiment number 14 :

Objective of experiment : to establish normal values for Allen's heparin-protamine titration test.

Technique : described on pages 35, and 36, variation b.

Solutions : Heparin Connaught and P_{A2}, containing 100 gamma of protamine in 0.1 cc. of distilled water.

Results : are shown in table XVII and in figures 6A and 6B.

Comment : When the first tests were done with Allen's method, it was noted that the blood in the tubes had clotted long before 60 minutes. Therefore, the tubes were inspected for clotting at shorter intervals. It can be seen from figure 6A, that clotting takes place first in the tube containing the largest amount of protamine, and later in the tubes containing smaller amounts of protamine. One or two tubes, containing 100 and 120 gamma of protamine respectively, remain unclotted even after 24 hours. It can also be seen, that the slope of the curves is not the same in all tests. According to Allen's method, the "heparin-protamine equivalents", read at 60 minutes, are (see figure 6B):
Heparin/protamine = 100/120 in one case, and heparin/protamine = 100/140 in 4 cases (in figure 6B only one of each group is represented. F= Fluid, C = clotted. The dotted line gives the 100/120 equivalent, the drawn line the 100/140 equivalent).

But, it is obvious, that 1) inspection of the tubes at 60 minutes does not give any information about the shortest clotting time in the series. The shortest clotting time is less than 15 minutes and takes place in the tube with 180 or 200 gamma of protamine.

2) the smallest amount of protamine that restores the coagulability of heparinized blood after 60 minutes, is not the smallest amount of protamine which neutralizes the anticoagulant effect of

heparin. The amount of protamine that neutralizes the effect of heparin is approximately 180 to 200 gamma of protamine.

3) Hence, the heparin-protamine equivalent is not 100/120, but 100/180 or 100/200. The equivalent cannot be read accurately, because the clotting times are only followed at large intervals.

4) What, then, is the evaluation of an arbitrary reading (at 60 minutes) of such a heparin-protamine titration? It gives information about the coagulability of the blood in a tube, containing a certain amount of protamine and an amount of heparin, which is partially neutralized by the protamine. In other words, it gives information about the coagulability of the blood in a tube containing an excess of heparin. If the whole heparin-protamine titration curve were completed, it could be seen that the curves, pictured in figure 6A and 6B, form the left limbs of the V-shaped heparin-protamine titration curves. The shape of the curve in Allen's test is not V-like but more U-like, due to certain peculiarities of the test.

5) Further observations are: the blood in the tubes is hemolysed, due to the dilution with water, and probably also due to the handling of the blood.

6) The clotting times are very short. Even at room temperature, which has a prolonging effect on the clotting time. The addition of water has a shortening effect on the clotting time of heparinized blood and also the handling of the blood may shorten the clotting time.

7) The slope of the different curves in figure 6A varies, so that in one instance the "equivalent" at 60 minutes is read as 100/120 (see figure 6B) and in four instances as 100/140. There is thus a certain variability possible, and a shift of 100/120 to 100/140 is within the range of normal variation.

In figures 6A and 6B some results with solution P_{A2} are represented. Other tests with different solutions have been carried out, which showed the same pattern. In Table XVII the reports of the tests presented in figure 6A are given.

(1)

TABLE XVII

F = fluid blood
C = clotted blood

Each tube contains 100 gamma heparin + 1 cc. blood, plus:

	Protamine Gamma/tube	100	120	140	160	180	200
Inspected for clotting at:	15 Min.	F	F	C	C	C	C
	30 "	F	C	C	C	C	C
	45 "	F	C	C	C	C	C
	60 "	F	C	C	C	C	C
	120 "	F	C	C	C	C	C
	24 hrs.	F	C	C	C	C	C

(2)

	Protamine gamma/tube:	100	120	140	160	180	200
Inspected for clotting at:	15 min.	F	F	F	C	C	C
	30 "	F	F	F	C	C	C
	45 "	F	F	C	C	C	C
	60 "	F	F	C	C	C	C
	120 "	F	C	C	C	C	C
	24 hrs.	F	C	C	C	C	C

(3)

Each tube contains 100 gamma heparin + 1 cc. blood, plus :

	Protamine gamma/tube :	100	120	140	160	180	200
Inspected	15 min.	F	F	F	F	F	C
for	30 "	F	F	C	C	C	C
clotting	45 "	F	F	C	C	C	C
at :	60 "	F	F	C	C	C	C
	120 "	F	F	C	C	C	C
	24 hrs.	F	F	C	C	C	C

(4)

	Protamine gamma/tube :	100	120	140	160	180	200
Inspected	15 min.	F	F	F	F	C	C
for	30 "	F	F	C	C	C	C
clotting	45 "	F	F	C	C	C	C
at :	60 "	F	F	C	C	C	C
	120 "	F	F	C	C	C	C
	24 hrs.	F	F	C	C	C	C

(5)

	Protamine gamma/tube :	100	120	140	160	180	200
Inspected	15 min.	F	F	F	F	F	C
for	30 "	F	F	F	C	C	C
clotting	45 "	F	F	C	C	C	C
at :	60 "	F	F	C	C	C	C
	120 "	F	F	C	C	C	C
	24 hrs.	F	F	C	C	C	C

Experiment number 15:

Objective of experiment: to determine the influence of heparin and dicumarol therapy on Allen's test, read at frequent intervals.

Technique: described on pages 35 and 36. For the estimation of the prothrombin times, the bedside technique was used. (p.39).

Solutions: Heparin Connaught and P_{A2}, containing 100 gamma of protamine in 0.1 cc. of distilled water.

Results: are shown in table XVIII and in figures 6C and 6D.

Comment: The normal values for Allen's test are established in experiment 14.

The patient was a young woman with phlebothrombosis. She was put on heparin intramuscularly, 50 mgm. every 4 hours, and an initial dose of 200 mgm. dicumarol was given (see table XVIII).

The protamine-heparin titration tests, performed before and during therapy, showed the following changes: Curve 1, before therapy, is flat. Under the influence of heparin the left limb of the curve first steepens (curve 2), and later the curve not only shows a steeper slope, but starts at a higher level (curve 4).

A reverse movement takes place after heparin therapy is discontinued. When the tests are read after 60 minutes only (Allen's method), a shift of the "equivalent" from 100/120 to 100/140 is noticed in curves 3,4 and 5. This shift does not exceed the variation found in normals. It is not possible to define sharply the heparin and the dicumarol influence on the titration test in this experiment, but it is certain, that heparin alone exerts its influence on curve 2 and that heparin and dicumarol together influence curve 4. Thus, under the influence of heparin the shape of the curve has changed, but the "equivalent" between heparin and protamine in Allen's test has not changed. Heparin and dicumarol together change the curve

further, and also cause a change in the "equivalent" in Allen's test from 100/120 to 100/140.

TABLE XVIII

Date: 1949	Dicumarol (mgm.)	Heparin i.m. (mgm.)	Hour of heparin injection	Time of blood test.	Allen's heparin/ protamine equiv. (at 60', R.T.)	Curve	C.T. 37°C. (min)	Prothr. time (sec.)
June 14	200	50	2 p.m.	1 p.m.	100/120	(1)		16
		50	6 p.m.					
		50	10 p.m.					
June 15		50	2 a.m.					
		50	11 a.m.	10.45 a.m.	100/120	(2)		
	100	50	2 p.m.					17
		30	6 p.m.	3.45 p.m.	100/140	(3)		
		30	10 p.m.					
June 16		30	2 a.m.					
		30	10 a.m.					
				10.45 a.m.	100/140	(4)	60'	35
				4.15 pm.	100/140	(5)	30-45'	
June 17				10. a.m.	100/120	(6)	20'	40
June 18	100							22
June 20	150							20
June 21	100			10 a.m.	100/120	(7)		20

Experiment number 16

Objective of experiment: 1) to determine the heparin-protamine equivalent in two modifications of Allen's test, a) using a waterbath at 37°C., and b) using a saline dilution and a waterbath at 37° C.

2) to determine the heparin-protamine equivalent between H_4 and $P_{A2\ dil.}$ in Jaques' test.

Technique: 1) for the modifications of Allen's test, see page 35 and 36, variations b and c.

2) for Jaques' test, see page 34.

The blood was taken with siliconed needle and syringes.

Solutions: Heparin Connaught, and P_{A2}, containing 100 gamma of protamine in 0.1 cc. of distilled water, are used for Allen's test. H_4, containing 10 gamma of heparin in 0.1 cc. of saline, and $P_{A2\ dil.}$ containing 10 gamma of protamine in 0.1 cc. of saline, are used for Jaques' test.

Results: see table XIX, a, b, and c, and figures 7A and 7B.

Figure 7A and Table XIX a, show the influence of the use of a waterbath at 37°C. on Allen's test. The clotting times are shortened, but otherwise the shape of the curve is the same. The heparin-protamine equivalent in this test is 100/180 in the highest curve, and in the lower curve 100/140. However, it is believed that the lower curve is "flattened" due to some influence in vitro or in vivo that increases the blood coagulability.

Figure 7B and Table XIX B show the influence of the saline dilution and the use of a waterbath at 37°C. on Allen's test. It can be seen that the anticoagulant influence of protamine is greatly increased by the use of saline as a diluent. The curve is more V-shaped, the heparin-protamine equivalent is easier to read than in figure 7A. It is 100/140 in two instances.

The heparin-protamine equivalent between H_4 and a 10 times diluted solution of P_{A2}, gives a value of 10/12 in three instances. (table XIX C). The $P_{A2\ dil.}$ solution has therefore the same strength and the same heparin-equivalent value as the P_4. The difference in heparin-protamine equivalents in the three tests (Tables XIX, A, B, and C, and figures 7A and 7B) cannot be ascribed to differences in the protamine, but are due to the different circumstances of the tests: for instance,

1) the use of very large amounts of heparin and protamine (figure 7B, compare figure IA), and 2) in addition to that, the dilution with water (figure 7A), which causes a further shift of the equivalent to higher protamine values, and 3) shortening of the clotting times gives a pseudo shift to lower protamine values. (figure 7A).

TABLE XIX (A)

Each tube contains 100 gamma heparin + 0.1 cc. distilled water + 1 cc. of blood, plus :

Protamine gamma/tube	100	120	140	160	180	200
	CLOTTING TIMES (Min.) at 37°C.					
Mrs. D.	>60	7	4	4	4	4
Mrs. L.	>30	10	8	7	6	7

(B)

Each tube contains 100 gamma of heparin + 0.5 cc. saline + 0.5 cc. blood, plus :

Protamine gamma/tube	100	120	140	160	180	200
	CLOTTING TIMES (Min.) at 37°C.					
Miss C.	>90	10	8	9	14	22
Dr. R.	>60	16	10	10	27	33

(C)

Each tube contains 100 gamma of heparin + 0.5 cc. saline + 0.5 cc. blood, plus :

Protamine gamma/tube P_{A2} dil.	8	10	12	14	16	Control. 0.5 cc. saline + 0.5 cc. blood.
	CLOTTING TIMES (Min.) at 37°C.					
Miss S.	30	13	12	12	16	11
Dr. R.	24½	10½	9½	12	11½	9½
Mrs. M.	29 p.c.	10½	10	11½	11½	11

Experiment number 17 :

Objective of experiment : to compare the three modifications of Allen's test in normals and in patients with blood dyscrasias.

Technique : described on pages 35 and 36.

Solutions : Heparin Connaught and P_{A3}, containing 100 gamma of protamine in 0.1 cc. of distilled water.

Results : see tables XX, XXI, XXII and XXIII and figures 8 A,B,C and D.

Comment : 1) Curves in normals : It can be seen from figures 8 A and C, that the curves differ in the test at 37°C. and in the test, using saline as a diluent, at 37°C. Comparison of the curves at 37°C with the curves at room temperature (see figure 6 A), show that elevation of temperature shortens the clotting times, so that a flat curve results, with a sudden angle towards incoagulability. The time lapse between venapuncture and performance of the test also has an influence on the clotting times. The longer the period between venapuncture and performance of the test, the shorter the clotting times, and, consequently, in figure 8A the angle in the curve shifts to the left, the later the test is performed after venapuncture.

Allen's test at room temperature gives a heparin-protamine "equivalent" at 100/120 in two cases and at 100/140 in one instance. (table XXII) This is comparable with former experiments (number 14).

If the test is performed with saline and at 37°C., the clotting times are longer, and the shape of the curve changes. The heparin-protamine equivalent is 100/160 in three cases.

2) Curves in blood dyscrasias : the test at 37°C (fig.8B) was carried out between 30 and 42 minutes after venapuncture.

Compared with the curve on normal blood, performed between 11 and 42 minutes after venapuncture, one can observe: 1) in some cases a higher level at which the curve starts, and 2) in some cases a steeper slope of the left limb of the curve.

In the test with saline, carried out at 37°C (figure 8 D) there is in 4 out of 6 cases a trend of the curve towards a higher level. The angle between the two limbs is sharper than in the control cases. The heparin-protamine equivalents are 100/160 in 4 cases, 100/140 in one case and 100/180 in one case. In the majority of the cases there is no shift of the equivalent. The changes in the protamine-heparin equivalent that occur may be due to technical errors.

The test at room temperature (table XXII) shows an "equivalent" at 100/140 in all cases. Some of the blood specimens containing 120 gamma of protamine were found to be clotted after 120 minutes.

The control clotting times in 4 of the 5 cases of blood dyscrasias was slightly prolonged (table XXII), and in the fifth case markedly prolonged. (Normal: 20-24 minutes). Three cases showed thrombocytopenia, and one case a prolonged prothrombin time. The most pronounced changes in the protamine-heparin tests occurred in case B, who also showed the greatest changes in the clotting time, prothrombin time and platelet count. Yet, the blood of this patient showed a change in Allen's test at room temperature, which did not exceed the variation encountered in normals.

TABLE XX

Each tube contains 100 gamma of heparin, 0.1 cc. of distilled water, 1 cc. of blood, plus:

Protamine gamma/tube:	100	120	140	160	180	200	Test performed.
NORMALS:	CLOTTING TIMES (Min) at 37°C.						
1	>24 hrs.	15	7	7	6	7	11 min. after v.P.
2	>24 "	10	7	7	7	9	21 " " "
3	>24 "	7	7	7	7	7	42 " " "
BLOOD DYSCRASIAS:							
Kr.	>24 hrs.	15	8 P.C.	7	7	7	41 min. after v.P.
L	>24 "	11	8	7	7	7	36 " " "
Ke.	>24 "	24	17	16	16	17	30 " " "
G.	>24 "	21	12	11	9	10	38 " " "
B.	>24 "	>24 hrs.	9	9	8	9	42 " " "

P.C. = Partial Clot

TABLE XXI

Each tube contains 100 gamma of heparin, 0.5 cc. of saline, 0.5cc. of blood, plus :

Protamine gamma/tube :	100	120	140	160	180	200	Test performed.
NORMALS :	CLOTTING TIMES (Min.) at 37°C.						
1. (Woman)	>24 hrs.	16	17	15	15	21	5 min. after V.P.
2. "	>24 "	12	12	11	18	32	18 " " "
3. "	>24 "	14	14	9	15	27	33 " " "
BLOOD DYSCRASIAS :							
M. (Man)	>24 hrs.	14	9	8	14	20	18 min. after V.P.X
Kr. "	>24 "	16	15	13	13	24	35 " " "
L. "	>24 "	-	27	16	16	24	30 " " "
Ke. "	>24 "	24	16	20	20	26	26 " " "
G. "	>24 "	30	24	21	20	30	30 " " "
B. "	>24 "	80 P.C.	27 P.C.	25	25 P.C.	34	37 " " "

P.C. = Partial clot

X - Venapuncture not clean, blood for only one test could be obtained.

TABLE XXII

	Allen-test R.T. (after 60 min.)			Control C.T. 37°C.: (min.)	Prothr. time (sec.)	Platelets per mm3.
NORMALS:	Gammas protamine/ tube. 100	120	140			
1.	F	F	C	22	-	-
2.	F	C	C	20	-	-
3.	F	C	C	-	-	-
BLOOD DYSCRASIAS:						
Kr.	F	P.C.	C.	32	15"	250,640
L	F	F	C	35	-	-
Ks.	F	F	C	30	15"	185,840
G.	F	P.C.	C.	33	19"	160,000
B	F	F	C	92	27"	89,960

P.C. = Partial Clot

TABLE XXIII

Name	Sex	Age	Diagnosis	Previous X-ray therapy	N_2-mustard therapy	Date of test - 1949
G.	M	40 y.	Reticulumcell Sarcoma (Gen. lymphadeno-pathy. Hepato- and splenomegaly.)	One course	4 courses 84 mgm. total Last inj. March '49	July 15
Ke.	M	40 y.	Hodgkin's disease (generalized lymphadeno-pathy)	-	2 courses 36 mgm. total Last inj. May '49	July 19
Kr.	M	33 y.	Hodgkin's disease (generalized lymphadeno-pathy)	8 courses	3 courses. 66 mgm. total Last inj. July 2/49	July 18
L	M	53 y.	Lymphosarcoma (generalized lymphadeno-pathy)	3 courses	2 courses. 50 mgm. total Last inj. July 2/49	July 16
M.	M	55 y.	Chron. lymph. Leucaemia.	-	-	July 14
B.	M	40 y.	Reticulumcell Sarcoma (Hemorrhages!) thrombocytopenia	Therapy: Blood transfus-ions		July 11
C.	M	37 y.	Polycythemia vera.	April 14 - May 16 210 r total body spray		
Mrs. M	F	59 y.	Monocytic Leucaemia (haemorrhages!) thrombocytopenia hypoprothrombin-emia (24"-27" Prothr.T.)	Febr.21-March 31/49 95 r spray	Aminopterin 10 mgm, from March 14-April 9/49	

Experiment number 18 :

Objective of experiment : to apply the heparin clotting test in some blood dyscrasias, and compare the results with other clotting test.

Technique : described on page 39 F).

Solution : R-solution, containing 4 gamma of heparin in 0.1 cc. of saline.

Results : are shown in table XXIV, for some data on patients see table XXIII.

Comment : The heparin clotting time is prolonged in case G, and in cases B, C. and Mrs. M. In Mrs. M. the heparin clotting time is not shortened after intravenous injections of protamine, except on one occasion. In case C. the heparin clotting time became markedly prolonged in the course of X-ray treatment.

TABLE : XXIV

(M) = Male
(F) = Female

Heparin γ :	4	4
Saline cc. :	0.10	0.10
Blood cc. ·	1.-	1.-
C.T. in min. at 37°C.		
Normal Range :	29-56	29-56
L. (M)	54	54
Ke. (M)	44	-
G. (M)	90 P.C.	-
B. (M) April 12/49	>9 hrs.	>9 hrs.
July 11/49	>24 hrs.	>24 hrs.
Mrs. M. (F) April 13/49	>3hrs.	>3 hrs.
May 17/49 (before)	>90	>90
19' after protamine injection	>90	>90
May 23/49 (before)	>180	-
15' after protamine injection	115	-
May 27/49 (before)	±150 P.C.	-
13' after protamine injection	±150 P.C.	-
C. (M) April 14/49	60	60
April 21/49	63	55
May 13/49	>120	>120
May 16/49 (before)	>105	>105
21' after protamine injection	>105	>105

Heparin γ ·	4	4
Saline cc. ·	0.10	0.10
Blood cc. ·	1.-	1.-
C. T. (Min. at 37°C.		
Normal Range :	29-56	
W. (M) Asthma Henoch-Schoenlein purpura	46	42
Co. (M) hypoplastic anemia	34	-
D. (M) bleeding without known cause	39	43

Experiment number 19 :

Objective of experiment : to apply the protamine test to some cases of blood dyscrasias.

Technique : described on page 37.

Solution : P_4, containing 10 gamma of protamine in 0.1 cc. of saline.

Results : see tables XXV and XXVI, and figures 9, A.B.C. and D and figure 10.

Comment : Cases Kr., L, Ke., G. W. Co. and D. show normal protamine curves. Cases B, C and Mrs. M, however, show curves in which the protamine sulfate prolongs the clotting time more than in normal cases (fig. 9). Protamine curves, carried out before and after the intravenous injection of 50 to 100 mgm. of protamine sulfate show in one instance a flatter curve after the injection; in a second case no difference before and after the injection (figure 9 D); and on two occasions a steeper curve after the injection.

(The protamine sulfate injected was a 1% solution of B.D.H.)

TABLE XXV

Protamine gamma :	-	-	2	4	6	8	10	12	16
Saline cc. :	-	0.50	0.48	0.46	0.44	0.42	0.40	0.38	0.34
Blood cc. :	1.00	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
CLOTTING TIMES (Min) at 37° C.									
Normal range :	8½-15	7½-14	9½-16	11-17	12-20½	11½-24	12½-28½	14-26½	15-28
Kr.	17	14½	17	19	19	21	24	27	28
L.	22	18½	16½	22½	23	23½	27	26	29½
Ke.	20½	16½	18½	16	17	23	19½	23	22
G.	20½	13	19½	19½	22	22	23½	26½	30
B.(April 12/49)	17	14	16	19½	21	13	28½	32½	32½
July 11/49	32 P.C.	26	25	28½	29 P.C.	30 P.C.	54 P.C.	61	48 P.C.
July 15/49	58 P.C.	37	42	56	56	50 P.C.	64 P.C.	85	85
W. (Henoch-Schoenlein purpura)	8½	8½	12½	12½	16	17½	18½	26	27½
Co. (Hypoplastic anemia)	-	15	17	17½	19	20½	20½	23½	26
D (bleeding with unknown cause.	14½	10½	11	13	16	20	17½	18	20

TABLE XXVI

P.C. = Partial Clot.

Protamine gamma :	-	-	2	4	6	8	10	12	16
Saline cc. :	-	0.50	0.48	0.46	0.44	0.42	0.40	0.38	0.34
Blood cc. :	1.-	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50

CLOTTING TIMES (Min.) at 37°C.

Normal range :	8½-15	7½-14	9½-16	11-17	12-20½	11½-24	12½-28½	14-26½	15-28
C.(April 14/49)	18½	16½	21½	23	28	34½	31	36	48½
April 21/49	18	19½	24½	30 P.C.	42½	58	57	56	56
May 13/49	21	17	23	26	33	43	43	50	43
May 16/49	21	15	21	29	32½	48	64	53	72
21 Min. after 50 mgm. protamine inj.	22	18½	20	28½	33½	57	55	40½	72
Mrs. M. April 13/49	9	10	12	12½	13	19	24	24	> 36
May 17/49	14½	15	21	35	38	70	83	89	88
19' after 100 mgm. protamine i.v.	11	12½	15	17½	26	34	38	46	44
May 23/49	16½	23½	24½	31	30	44	57	69	85
15' after 100 mgm. protamine i.v.	26½	32	45	52	59	92	43	88 P.C.	>100
May 27/49	21	19½	23½	16	25½	51	50	76	49
13' after 100 mgm. protamine i.v.	35½	34½	29	40	51	90	97	106	108
60' after inj. of protamine	33	26	38	49	69	80	76	82	103

Experiment number 20:

Objective of experiment: to investigate the effect of dicumarolisation on Allen's test and its modifications.

Solutions: Heparin Connaught, and P_{A4} and P_{A5}, each containing 100 gamma of protamine in 0.1 cc. of distilled water.

Technique: described on pages 35 and 36. For prothrombin time technique see page 39 G).

Results: see tables XXVII a and b, and XXVIII a and b, and figures 11, A,B,C and D.

Comment: using P_{A4}: the venapuncture in the second normal was not clean, which influenced the tests. The clotting times are shorter, so that a flat curve results in figure 11 A, and the "equivalent" in Allen's test, at room temperature, becomes 100/100.

In the modified test, using saline as a diluent and a waterbath at 37°C, the heparin-protamine equivalent is 100/160 in two instances and 100/140 in one instance (figure 11 C). This variation is probably due to technical errors.

The tests with blood of dicumarolised patients show the following differences: 1) the curves (in figure 11A) are on a higher level due to the prolongation of the clotting times.

2) the curves are somewhat steeper (fig. 11 A) and reach incoagulability earlier.

3) the "equivalent" in Allen's test is shifted to 100/140.

4) In one case there is a shift of the heparin-protamine equivalent (in figure 11 C) to a higher protamine value, but this may be due to a technical error.

using P_{A5}: Only one control value is used.

The curves with blood of dicumarolised patients show the following: 1) the curves in figure 11 B are on a somewhat higher level than the control curve.

2) The heparin-protamine equivalent in figure 11 D is the same as for the normal (100/160).

3) The "equivalent" in Allen's test at room temperature may, or may not, change from 100/120 to 100/140.

TABLE XXVII a

Each tube contains 100 gamma of heparin + 0.1 cc. distilled water + 1 cc. blood, plus:

Protamine gamma/tube:	100	120	140	160	180	200	100	120	140	
Normals:	CLOTTING TIMES (Min) at 37°C.						CLOTTING AFTER 60 Min. at Room Temperature.			
1. (F)	>180	6	6	5	5	5	F	C	C	
2. (F)	>60	4	3	2½	2	2½	C	C	C	
3. (M)	>120	7½	4½	4	4½	4½	F	C	C	
4. (M)	>24 hr.	9½	5	4½	4½	4	F	C	C	
DICUMAROLISED PATIENTS										Prothr. time (sec.)
Mr. B.	>24 hr.	>24 hr.	9	8½	7½	6½	F	F	C	95
Mrs. P.	>24 hr.	>24 hr.	11½	9	-	7	F	F	C	50

TABLE XXVII b.

Each tube contains 100 gamma of heparin + 0.5 cc. saline + 0.5 cc. blood, plus :

Protamine gamma/tube :	100	120	140	160	180	200	
Normals :	CLOTTING TIMES (Min) at 37°C.						
1. (F)	>180	6	5	6	7	12	
2. (F)	>60	6	4½	4	5	7	
3. (M)	>120	12	8	7	8½	12	
DICUMAROLIZED PATIENTS :							Prothr. time (sec.)
Mr. B. (Coronary thrombosis)	>24 Hr.	16	11½	11½	9	11½	95"
Mrs. P. (phlebo-thrombosis)	>24 hr.	15	12	11½	11½	16½	36"

TABLE XXVIII a.

Each tube contains 100 gamma of heparin + .1 cc. of blood + 0.1 cc. dist. water, plus :

Protamine gamma/tube :	100	120	140	160	180	200	100	120	140	Prothr. time (sec.)
Normal :	CLOTTING TIMES (min) at 37°C.						CLOTTING AT R.T. AFTER 60 min.			
1. (F)	>24 hr.	>24 hr.	12	6	5½	5½	F	C	C	
DICUMAROLISED PATIENTS :										
Mrs. W. (phlebo-thrombosis)	>24 hr.	>24 hr.	9	7	7	7	F	F	C	30"
Mrs. W.	>24 hr.	>24 hr.	16½	11½	11	11	F	F	C	47"
Mrs. L. before therapy (phlebo-thrombosis)							F	C	C	18"
Mrs. L.							F	C	C	43"
Mrs. B. (dicumarol therapy, phlebo-thrombosis)							F	C	C	49"
Mrs. B.							F	F	C	43"
Mrs. B.							F	F	C	58"

TABLE XXVIII b.

Each tube contains 100 gamma of heparin + 0.5 cc. saline + 0.5 cc. blood, plus :

Protamine gamma/tube :	100	120	140	160	180	200	Prothr. time (sec.)
Normals :	CLOTTING TIMES (Min) at 37°C.						
1. ( )	>24 hr.	25 P.C.	12	10	10	13½	
DICUMAROLIZED PATIENTS :							
Mrs. W.	>24 hr.	12	12	9	11	14	30
Mrs. W.	24 hr.	15	13	12½	12½	13	47

Experiment number 21:

With the Beckman p_H meter the following determinations were done:

Date	Solution:	p_H:
May 30/49	P_4	6.89
June 25/49	P_{A_2}	6.25
Dec. 6/49	P_{A_5}	6.50
May 30/49	0.05 cc. tricresol-isomers in 50 cc. of saline	5.90 5.85
Aug. 7/50	D_2 (same constitution as D_1)	6.79
	dextrose-citrate mixture (vacoliter)	6.0
	bank blood (dextrose-citrate blood)	6.32
May 30/49	normal saline	6.9
	Dilutions of saline with P_4 show decreasing p_H values:	
	A mixture of 0.16 cc. P_4 and 0.34 cc. saline has a p_H of: (Determinations of 2 different mixtures)	5.8 5.67

CHAPTER VII

Discussion

Chargaff (108), and later Jaques (99), showed that in a series of tubes, containing equal amounts of heparinized blood and increasing amounts of protamine, the clotting time first decreases with increasing amounts of protamine until it reaches a minimum, and then increases again due to the anticoagulant effect of protamine. In the tube with the shortest clotting time, the anticoagulant effect of heparin is neutralized by the protamine. The ratio of heparin and protamine at the point of neutralization (equivalence point) is constant for a certain heparin and protamine solution, but different for different solutions.

The equivalence point is formed by the angle in the v-shaped curve of the clotting times. When the angle is sharp, the equivalent can be read accurately. Thus, the accuracy of this method for the determination of small amounts of heparin in the blood depends upon the sharpness of the angle between the two limbs of the curve. What are the factors that determine the shape of the curve and thus the accuracy of this method of heparin determination?

1) the anticoagulant potency of the heparin and protamine preparations, used for the standard solutions, is of importance for the estimation of the heparin-protamine ratio of a certain protamine solution. The Connaught preparations are of great purity and potency.

2) Factors influencing the length of the clotting times. In one instance (Exp. 7, page52) it was found that the angle was sharper, when silicone technique was used, but this method was not further investigated. On the other hand, a technique which shortens the clotting times, as for instance bad venapuncture and extensive

handling of the blood in Allen's technique (exp. 14, p.69), will change the V-shape of the curve into a U-shape. Consequently, accurate reading of the equivalent has become impossible. Thromboplastin admixture, as in LeRoy's technique (131,132) makes it necessary to read the clotting times in seconds. LeRoy claims to be able to demonstrate 3.3 gamma of heparin per cc. of oxalated plasma. This method was not used in the present investigation. The accuracy of this method will depend on the technique of prothrombin time determination used.

3) The temperature has an influence on the curve. Room temperature prolongs the clotting times, but is otherwise too unreliable to be useful.

4) The technique of clotting time determinations will also determine the exactness of the method of heparin-protamine determinations. The variation in the determination of clotting times, as demonstrated in exp. 3, p. 44, increases when anticoagulant solutions are mixed with the blood (due to errors in measurement). This applies even more to the determination of heparin levels in the blood after intravenous injection of heparin, because the blood now contains the anticoagulant and has to be pipetted rather rapidly. (exp. 11 page 60).

5) the medium in which the protamine solution is dissolved plays an important part. Saline has been proven useful by Jaques. Water, on the other hand, is known to shorten the clotting time of heparinized blood (62) and should be avoided. (see also experiment 16, fig. 7 A and B).

6) The accuracy with which heparin and protamine can be measured also determines the exactness of the method.

7) The difference between the amounts of protamine in the consecutive

tubes is of the greatest importance. Although relatively large intervals (10 to 20 gamma) of protamine give a sharp angle in the curve, this does not improve the accuracy of the measurement of heparin, due to the fact that the angle of the curve may be anywhere in between the amounts of protamine contained in the tubes. When the amounts of protamine in the tubes differ 2 gamma, the angle of the curve becomes wider, and the exact equivalent point is difficult to determine, especially when in vivo heparin levels have to be determined. 8) The in vivo coagulability of the blood will determine the clotting times, and thus the shape of the curve. This point will be dealt with in detail later.

What factors determine the protamine-heparin equivalent?

1) The heparin-protamine ratio may be different for different solutions of heparin and protamine, and each batch has to be tested. 2) As Jaques (78) has shown, several factors will influence the dissociation of the heparin-protamine compound, and thus the equivalent, as there are: changes in p_H, addition of anions and cations, addition of proteins (haemoglobin), and lipoids. These factors interfere, because they (anions and cations and p_H) change the dissociation constant of the heparin-protamine compound or compete (proteins) for the available heparin or protamine.

In exp. 8, p. 54, the addition of a small amount of the dye, T-1824, caused the heparin-protamine equivalent to shift to higher protamine values. This was unexpected, because it was thought, that toluidine blue would interact with heparin and less heparin would be available to combine with protamine. This theory did not explain the observed facts. It might be that toluidine blue competed for the blood proteins (179), so that more heparin was available, or

that the addition of T-1824 changed the dissociation constant of the heparin-protamine complex. (Due to p_H changes? The p_H of a T-1824 solution of the same strength was 6.79). This cannot be decided without further investigation. In the test with the dextrose-citrated bank blood, it is conceivable that a p_H change took place (the p_H of the dextrose-citrate mixture is 6 as measured in one instance), which might have decreased the dissociation constant of the heparin-protamine complex. The influence of dextrose has not been investigated, and only suggestions can be made about the reason of the equivalent shift. The important fact is, however, that such changes take place. The influence of the proteins seems important in the determination of heparin-protamine equivalents in hypoproteinemic patients. Interesting also is the fact that water, according to Jaques, is responsible for the dissociation of the heparin-protein complex in heparinized blood, resulting in a shortening of the clotting time. It is thus not inconceivable that addition of water also increases dissociation of the heparin-protamine compound, thus requiring more protamine to combine with the heparin,(causing a shift of the heparin-protamine equivalent in Allen's test.) It was also found in test 1 and in experiment 16, that the heparin-protamine equivalent is not the same for dilute and concentrated heparin solutions. With large amounts of heparin (100 gamma per tube) the equivalent shifts to larger protamine values (100/160 instead of 100/120), as has also been shown by Holden (103). Thus, it is not justified to conclude from a shift in the heparin-protamine equivalent that more heparin is present in a blood sample, unless all other factors influencing the heparin-protamine equivalent are excluded. This applies to the heparin titration methods of Jaques and Allen, but in addition there is another factor, which has to be taken into account in Allen's method. That is, that in this

method, heparin is <u>added</u> to the blood, which is to be investigated for the presence of heparin or heparin-like substances. The amount of heparin added is high, 100 gamma per cc. of blood, which makes it doubtful whether small amounts of so called "endogenous" heparin (present in the blood before addition of the commercial heparin) can be detected. But there is something else. Due to the fact that the test is read at an arbitrary time, 60 minutes, and not at the shortest clotting time in the series, it is not the equivalent between heparin and protamine which is estimated, but the smallest amount of protamine necessary to clot blood containing 100 gamma of heparin <u>within 60 minutes</u>. In other words, the clotting time in the tube, inspected by Allen after 60 minutes, is situated on the left side of the U-shaped curve, and thus contains an amount of heparin, which is only partially neutralized by protamine. Thus the "equivalent" in Allen's test is read in a tube, containing an <u>excess of heparin</u>, and thus his test has the same significance as a heparin clotting test. The <u>heparin sensitivity</u> of the blood under investigation will determine the "equivalent" in Allen's test, as has been demonstrated in the experiments, where clot inspections were performed at intervals. The curves steepen in cases of decreased coagulability (blood dyscrasias; heparinization and dicumarolisation in certain instances) (exp. 14, 15, 17) and flatten in cases of decreased coagulability (due to in vivo factors, but also due to technical factors, as bad venapuncture (experiment 17) and delay of performance of the test.) In addition the <u>level</u> of the curve increases with decreased coagulability. It is therefore conceivable that thrombocytopenia, as suggested by Conley (42) has an influence on the outcome of the test. Lyle Carr and Fowler (41) showed in the Waugh-Ruddick test that the heparin sensitivity of

thrombocytopenic blood is increased. The fact that administration of heparin in vivo has little influence on the heparin-protamine equivalent in Allen's test and on the Waugh-Ruddick test also point to the same conclusion, that these tests do not measure heparin in the blood, but decreased coagulability, the cause of which is not identifiable by using these tests.

General hypothesis on the significance of heparin clotting tests, titration test of Allen, and protamine test:

It could be shown, that in certain blood dyscrasias protamine (exp. 19) had an anticoagulant effect on the clotting time far in excess of the anticoagulant effect in normals. Heparin tests in these cases showed the same effect. The prolongation of the clotting time by heparin was, however, longer, because heparin is a much more potent anticoagulant than protamine.

It is thus highly suggestive to apply the formula of Fischer (88) to the effects observed in these heparin and protamine clotting tests.

$\text{Log } T_2 - \log T_1 = k\,(c_2 - c_1)$ (see page 15). This formula was originally used to determine the potency of a certain heparin sample by Fischer. The factor k, which determines the angle formed by the straight line and the abscissa, indicates the heparin potency of the preparation used, if all other circumstances are constant. But, if the heparin potency is kept constant, the same formula can be used to indicate the heparin sensitivity of a blood sample (67). Thus, in cases of increased heparin sensitivity, the line will show a steeper angle, ($\log T_2 - \log T_1$) increases and k will increase, or: k is multiplied by a certain factor. This is the importance of heparin clotting tests for the discovery of decreased coagulability, that

heparin multiplies a factor in the blood, which is responsible for the decreased coagulability, no matter what that factor is. (With one restriction, that it does not neutralize heparin). Thus : heparin potentiates the effect of decreased coagulability on the clotting time. This decreased coagulability may be due to : platelet deficiency, prothrombin deficiency, or to any factor that decreases the coagulability, and also to any anticoagulant. However, the addition of small amounts of heparin does not have this effect. As can be seen from the formula, addition of small amounts of heparin to the tubes in a Waugh-Ruddick test on normal blood, will result in a curve parallel to the first curve, but on a little higher level (each tube now contains, for instance, as much heparin as in the next tube.) This is the addition of a constant value to all the clotting times of the curve. It explains why Theilen (87) found little influence on the Waugh Ruddick test in heparinized normal humans, in contrast to the counterclockwise change in thrombocytopenic patients. The same phenomenon was observed by Allen, and by this investigator. Heparin injections have little or no influence on the "equivalent" in Allen's test, but heparin plus a prothrombin deficiency will result in a shift. (addition versus multiplication of factors). This can be applied to protamine curves, which, as shown in experiment 19, did not neutralize any anticoagulant substances in the blood of patients with certain blood dyscrasias, but on the contrary potentiated the decreased coagulability of the blood of these patients. The curves were not very regular due to technical errors, but a counterclockwise shift could be observed. Thus, it seems, that heparin clotting time tests (and protamine clotting time tests) are useful to demonstrate decreased coagulability with no further indication of its nature or cause. One can conclude

that Allen's test, due to his addition of heparin in vitro, is simply a test for decreased coagulability, which shows no advantage over any of the other tests. LeRoy's test has to be further investigated, but the fact that the method is able to show heparin-like substances only if heparin is added makes it very likely that this test has the same significance as Allen's test. The value of heparin-protamine titration tests in the study of circulating anticoagulants can therefore be summarized as follows: Jaques' method, here described as the protamine test, seems the most useful one for demonstrating circulating anticoagulants with the characteristics of heparin, which combine with protamine. Its sensitivity, however, is limited and can possibly be increased by the use of silicone technique.

SUMMARY

1) Heparin-protamin titration tests, using Jaques' method were carried out and showed a rather constant heparin-protamine ratio, under constant circumstances. The ratio was found to be 10/12 for most of the solutions used. Variability in the ratio was due to technical errors.

2) The ratio changed when a T-1824 solution was added and when the test was carried out in recalcified dextrose-citrate blood. In concentrated heparin-protamine solutions the ratio was changed to higher amounts of protamine per gamma of heparin.

3) The smallest amount of heparin demonstrated by the method was 4 gamma of heparin added in vitro and 3.3 gamma of heparin added in vivo. Estimation of heparin added in vivo was less accurate than estimation of heparin added in vitro.

4) Accuracy of the method and factors influencing the method are discussed.

5) Normal values were established for the protamine test, and for the heparin clotting time method.

6) Allen's method of heparin-protamine titration was used and normal values established.

7) Allen's test was modified in three ways: A) inspection of clot formation at certain intervals demonstrated that the heparin-protamine equivalent was not read at 60 minutes and that the test could only be interpreted as a test for decreased coagulability.

B) A modification, using a waterbath at 37°C, was used, and

C) A modification, using a waterbath at 37°C, and saline as a diluent, was used. These methods were compared in normals and in patients with blood dyscrasias, and under heparin and dicumarol therapy.

8) It could be shown, that in the cases with blood dyscrasias no shift of the heparin-protamine equivalent took place, although Allen's test showed an "increased protamine titration" in some cases. This change did not exceed the normal range of variation of the test.

The "equivalent" in Allen's test was not influenced by heparin administration in vivo, but heparinization and dicumarolisation together showed a change in the "equivalent" as did dicumarolisation in some cases.

9) The protamine test and the heparin clotting time were markedly prolonged in some blood dyscrasias. Intravenous protamine sulfate did not neutralize anticoagulant substances as tested by the protamine test.

10) The importance of these findings is discussed and a hypothesis offered for the explanation of the action of prolonged clotting time tests, including Allen's test, in cases of decreased coagulability of the blood.

Conclusions:

1) Jaques protamine titration test, as it was used in this investigation, is probably not sensitive enough to demonstrate circulating anticoagulants of the heparin type in blood dyscrasias.

2) Allen's test is a test for decreased coagulability, and cannot show specifically the presence of heparin or heparin-like substances.

3) The presence of circulating coagulants of the heparin type can be demonstrated with certainty only by chemical methods.

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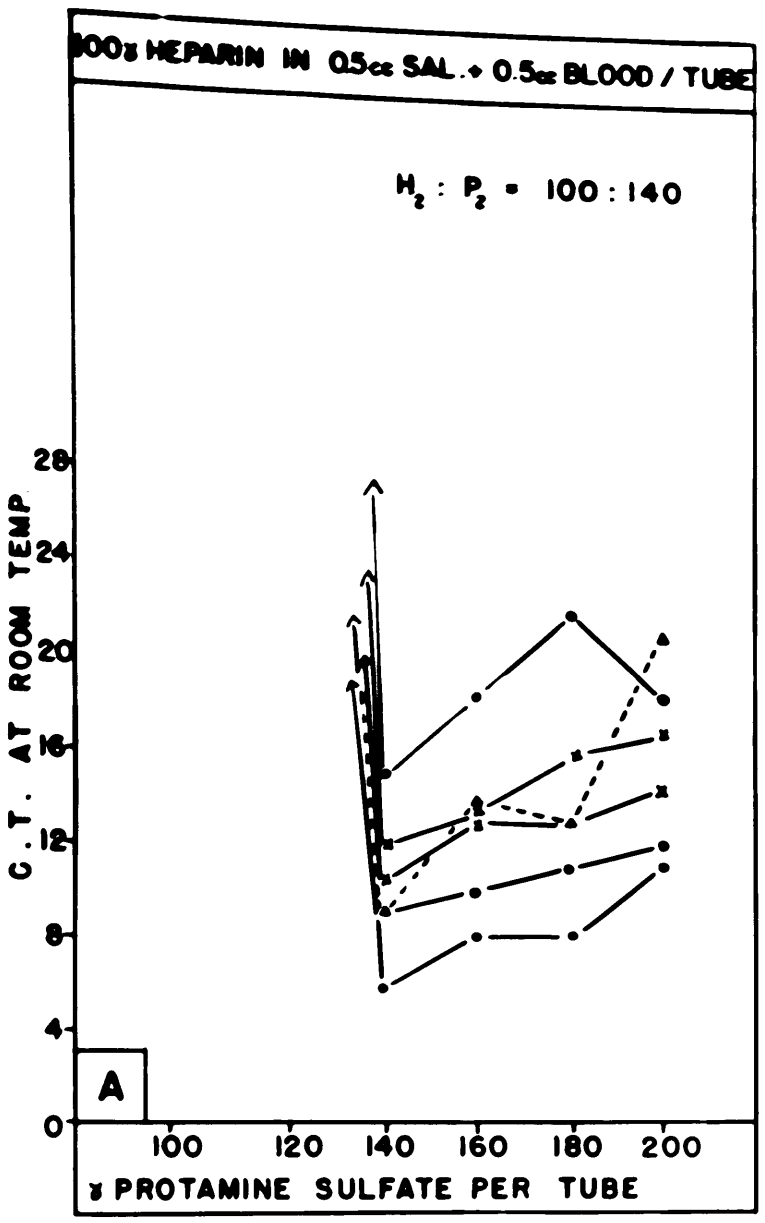
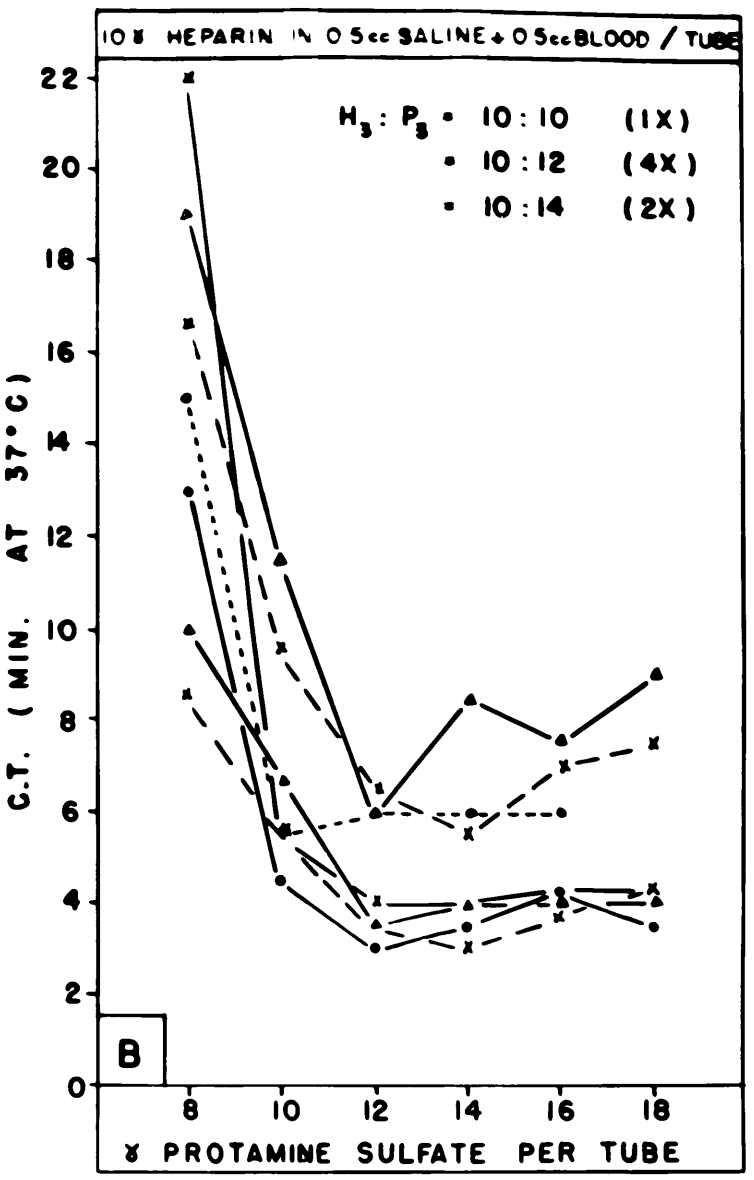
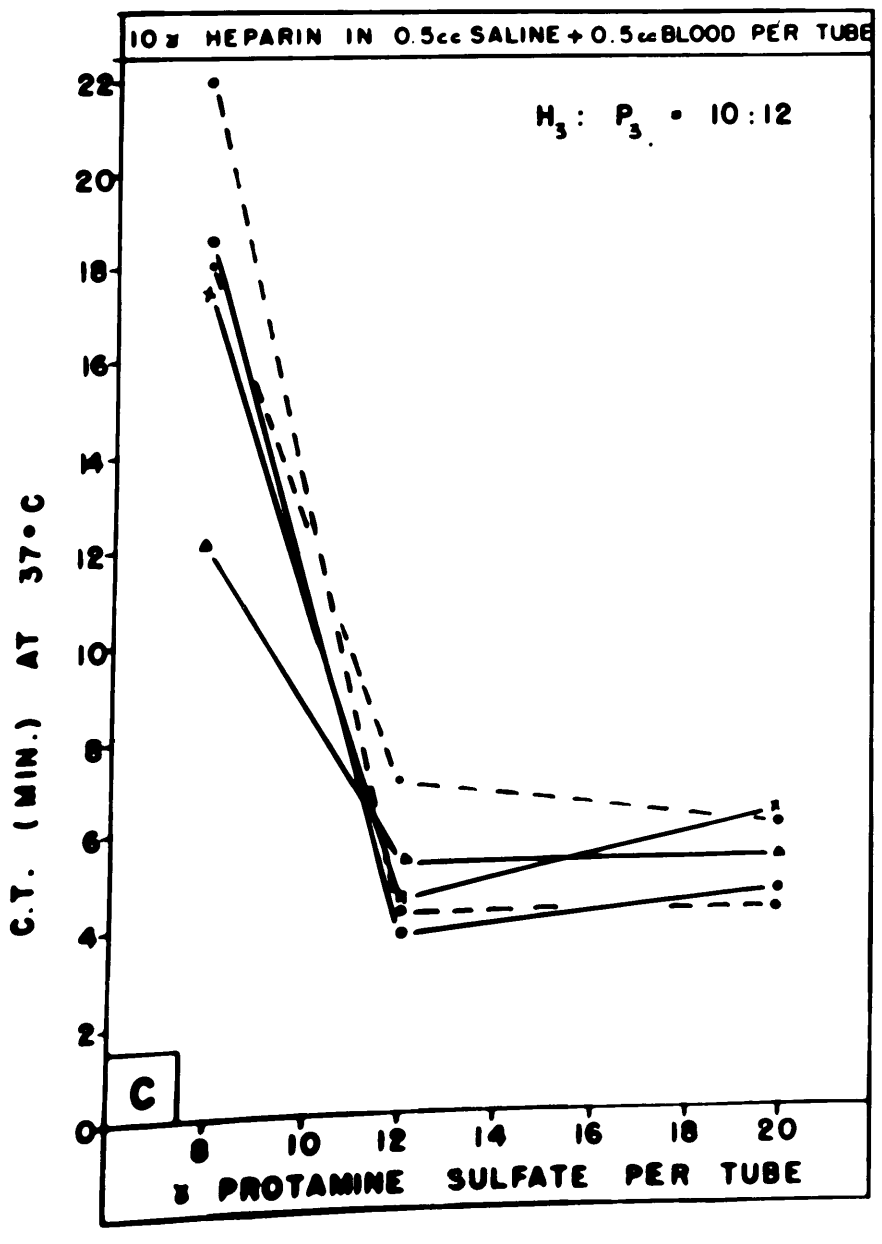

300 ɣ HEPARIN IN 0.5cc SAL. + 0.5cc BLOOD / TUBE
H_2 : P_2 = 100 : 140
C.T. AT ROOM TEMP
A
100 120 140 160 180 200
ɣ PROTAMINE SULFATE PER TUBE


10 ɣ HEPARIN IN 0.5cc SALINE + 0.5cc BLOOD / TUBE
H_3 : P_3 = 10 : 10 (1X)
= 10 : 12 (4X)
= 10 : 14 (2X)
C.T. (MIN. AT 37° C)
B
8 10 12 14 16 18
ɣ PROTAMINE SULFATE PER TUBE


10 ɣ HEPARIN IN 0.5cc SALINE + 0.5cc BLOOD PER TUBE
H_3 : P_3 = 10 : 12
C.T. (MIN.) AT 37° C
C
8 10 12 14 16 18 20
ɣ PROTAMINE SULFATE PER TUBE

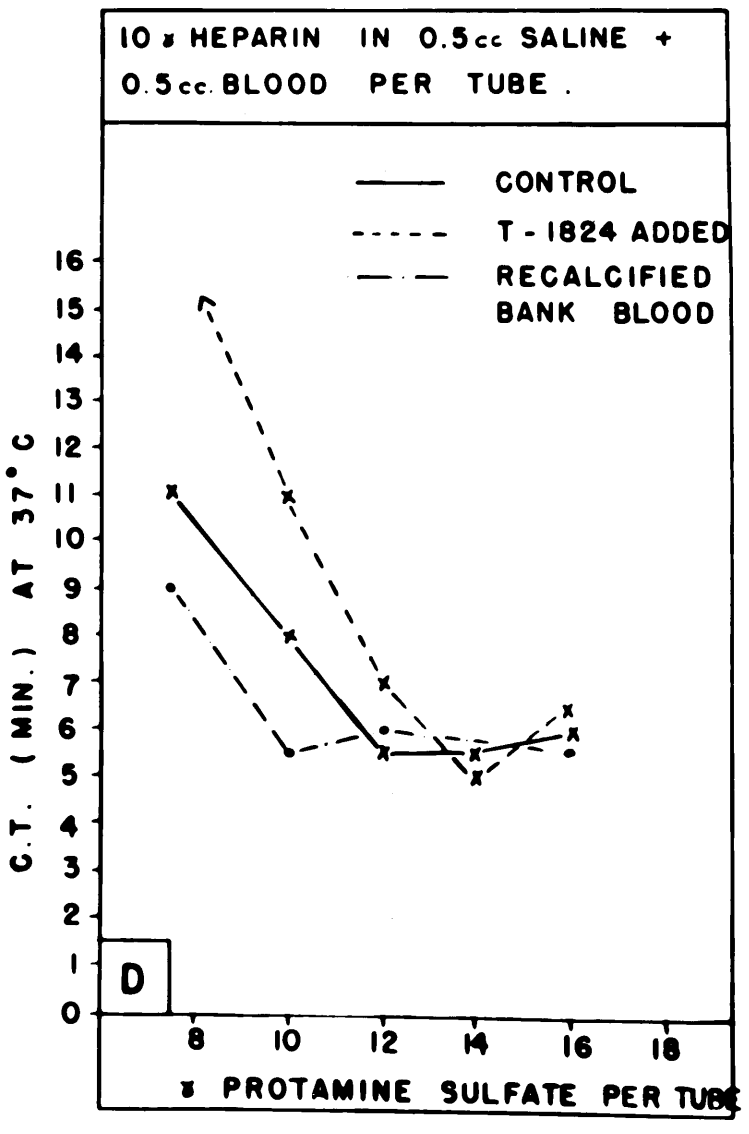

10 ɣ HEPARIN IN 0.5cc SALINE + 0.5cc. BLOOD PER TUBE .
CONTROL
T - 1824 ADDED
RECALCIFIED BANK BLOOD
C.T. (MIN.) AT 37° C
D
8 10 12 14 16 18
ɣ PROTAMINE SULFATE PER TUBE

Fig.2.

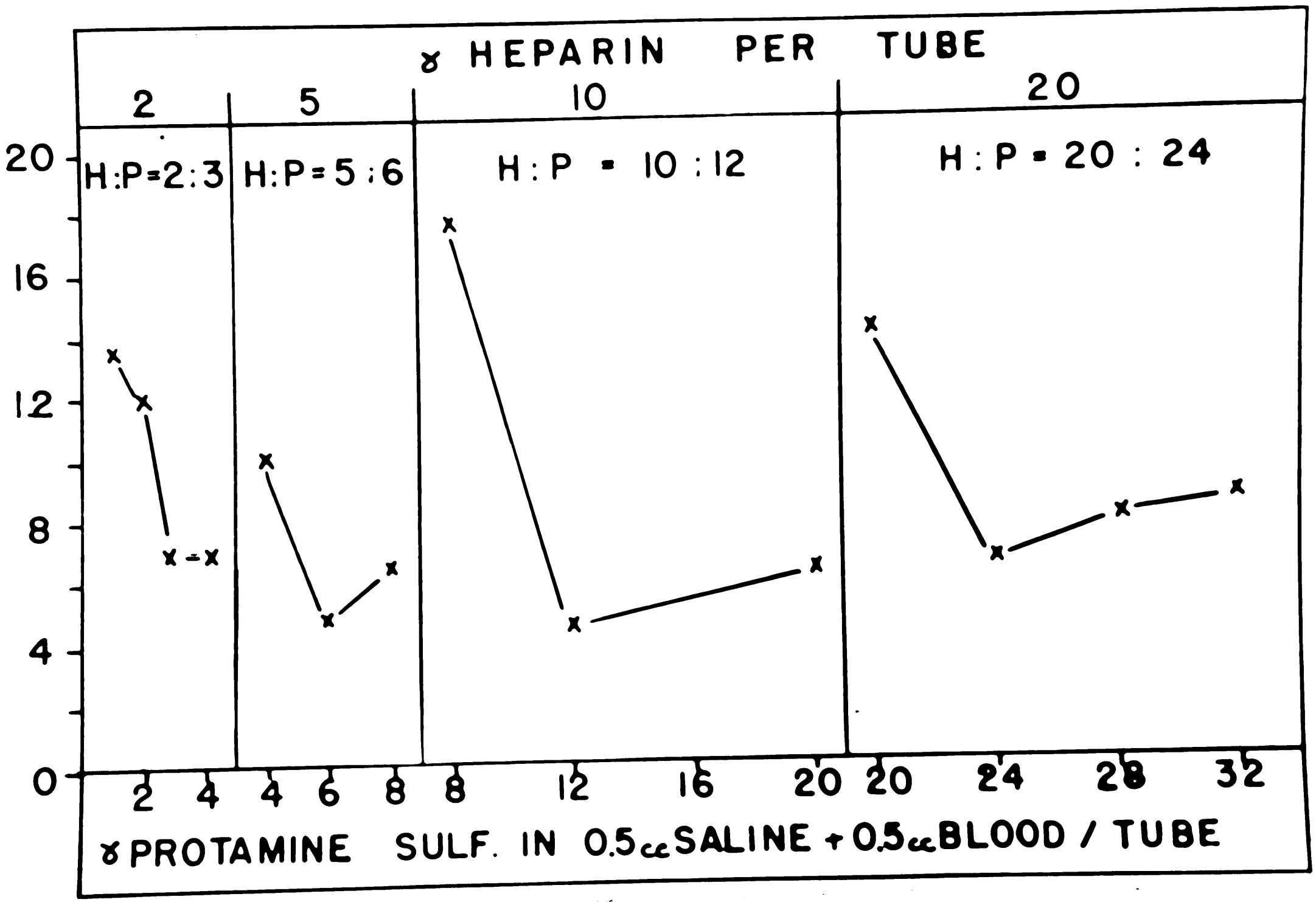

γ HEPARIN PER TUBE
2
5
10
20
H:P=2:3
H:P=5:6
H:P = 10:12
H:P = 20:24
20
16
12
8
4
0
2 4 4 6 8 8 12 16 20 20 24 28 32
γ PROTAMINE SULF. IN 0.5cc SALINE + 0.5cc BLOOD / TUBE

Fig.3.

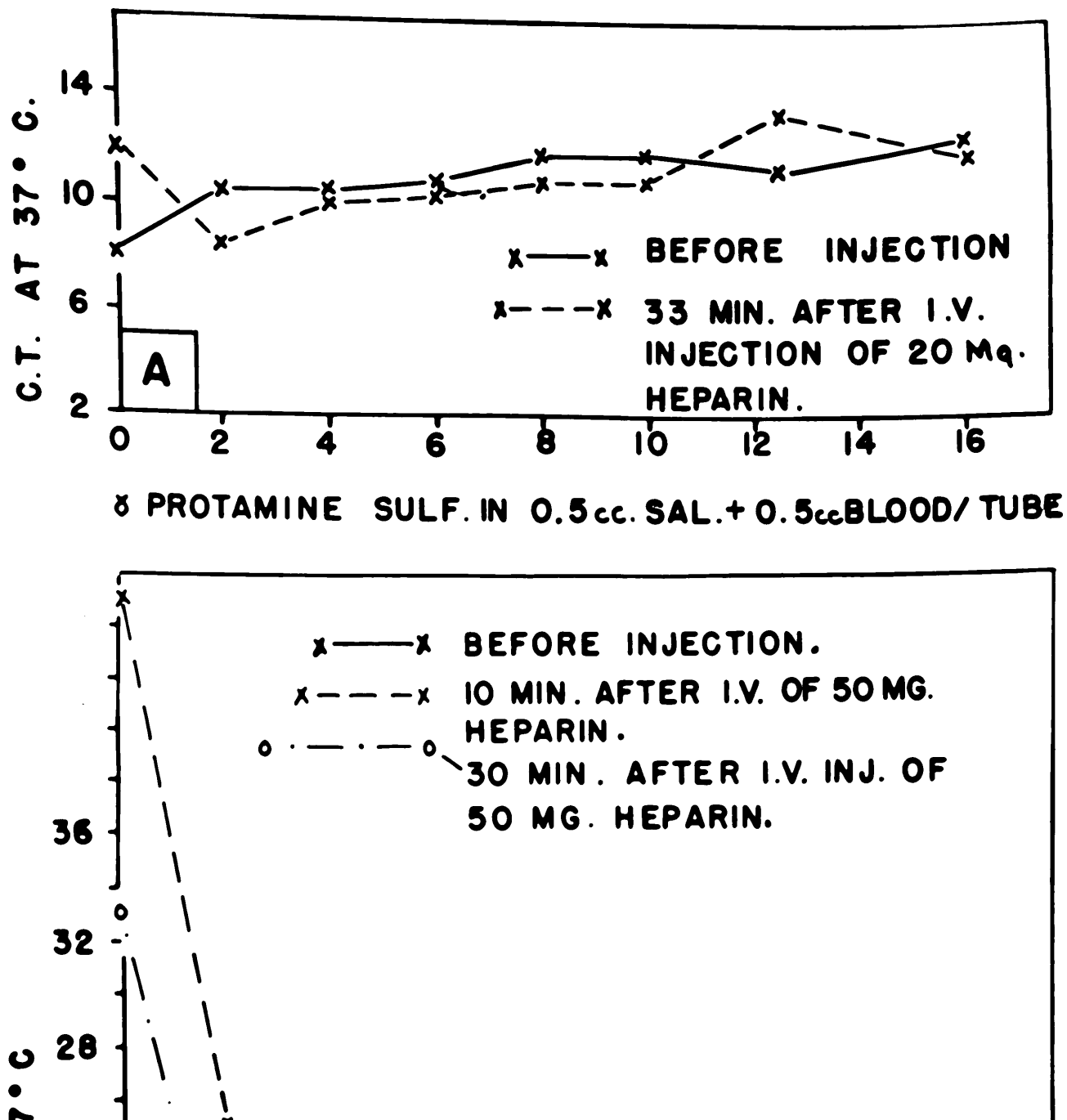

C.T. AT 37° C.
14
10
6
2
A
0
2
4
6
8
10
12
14
16
BEFORE INJECTION
33 MIN. AFTER I.V. INJECTION OF 20 Mg. HEPARIN.
γ PROTAMINE SULF. IN 0.5cc. SAL. + 0.5cc BLOOD/ TUBE
BEFORE INJECTION.
10 MIN. AFTER I.V. OF 50 MG. HEPARIN.
30 MIN. AFTER I.V. INJ. OF 50 MG. HEPARIN.
C.T. (MIN.) AT 37° C
36
32
28
24
20
16
12
8
4
0
B
2
4
6
8
10
12
14
16
γ PROTAMINE SULF. IN 0.5cc SAL. + 0.5cc BLOOD/ TUBE

Fig.4.

PROLONGATION OF CLOTTING TIME BY PROTAMINE SULFATE

(20 NORMALS)

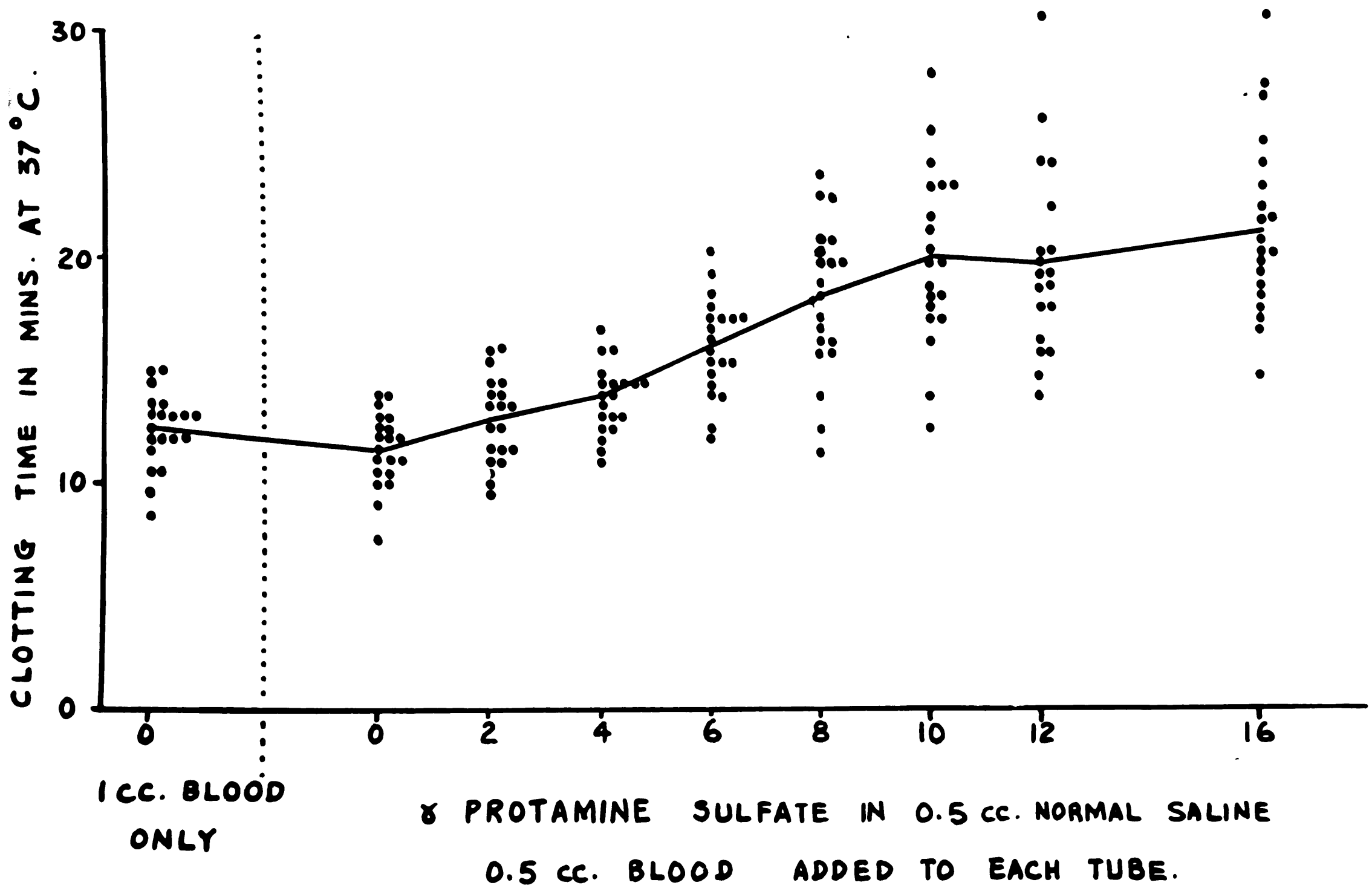


Fig.5.

HEPARIN CLOTTING TIMES (30 NORMALS)

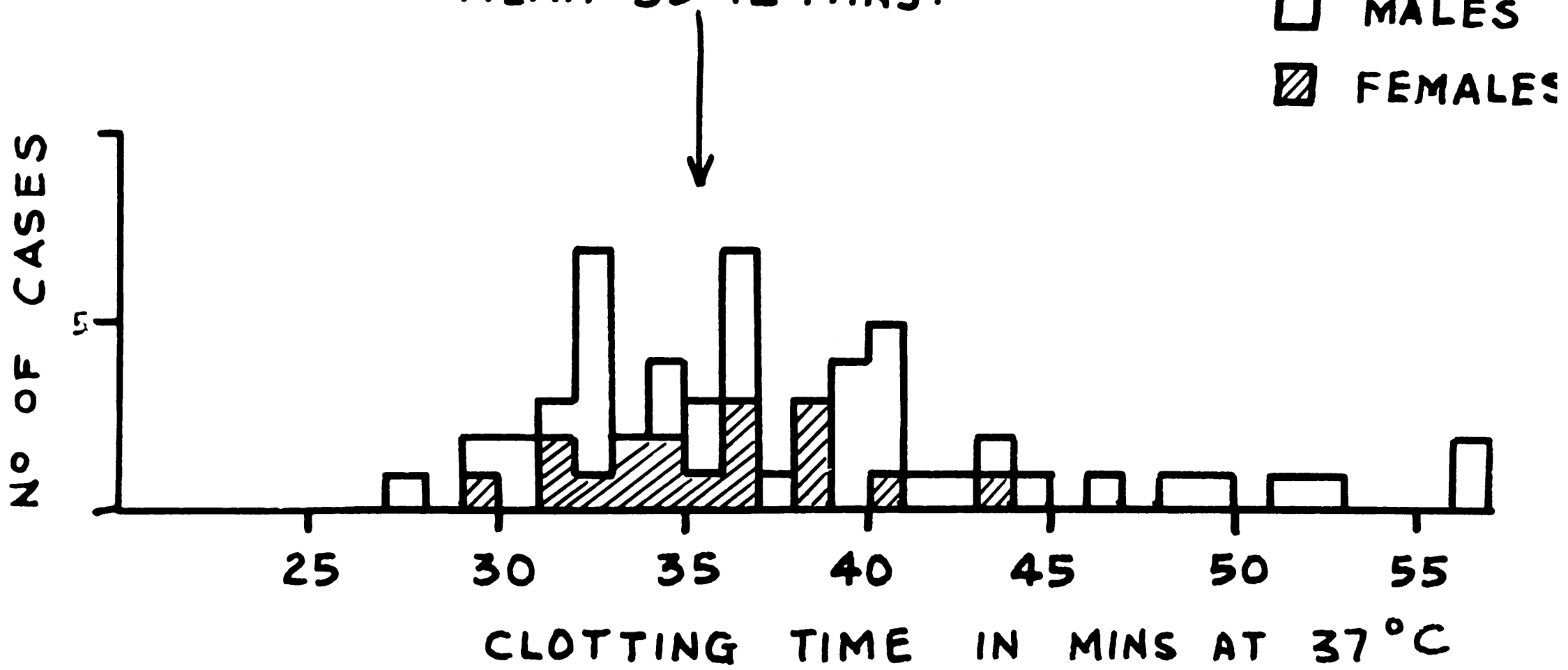
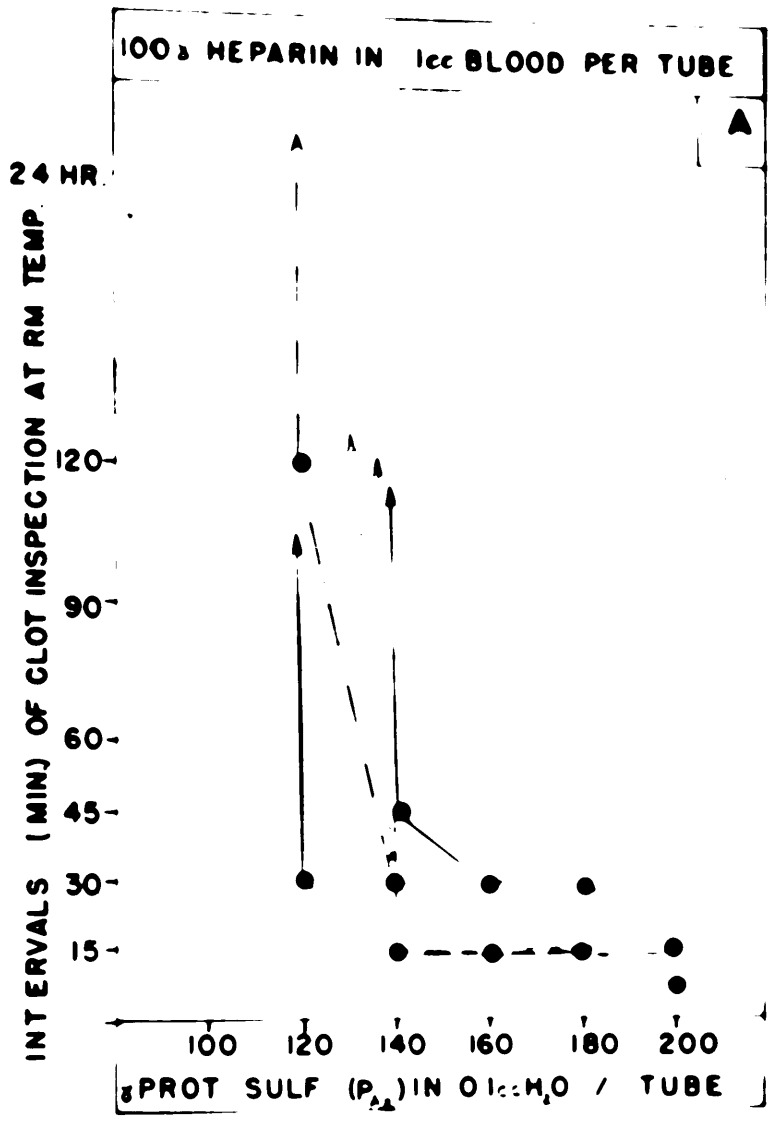
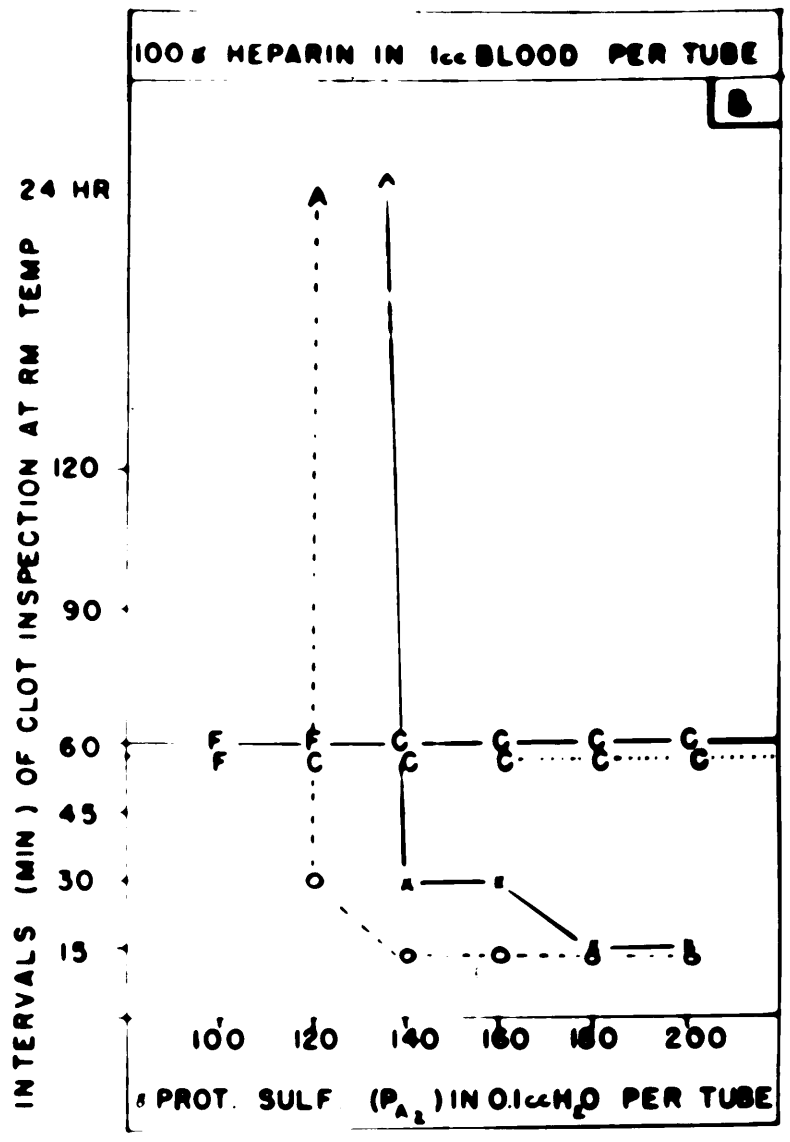
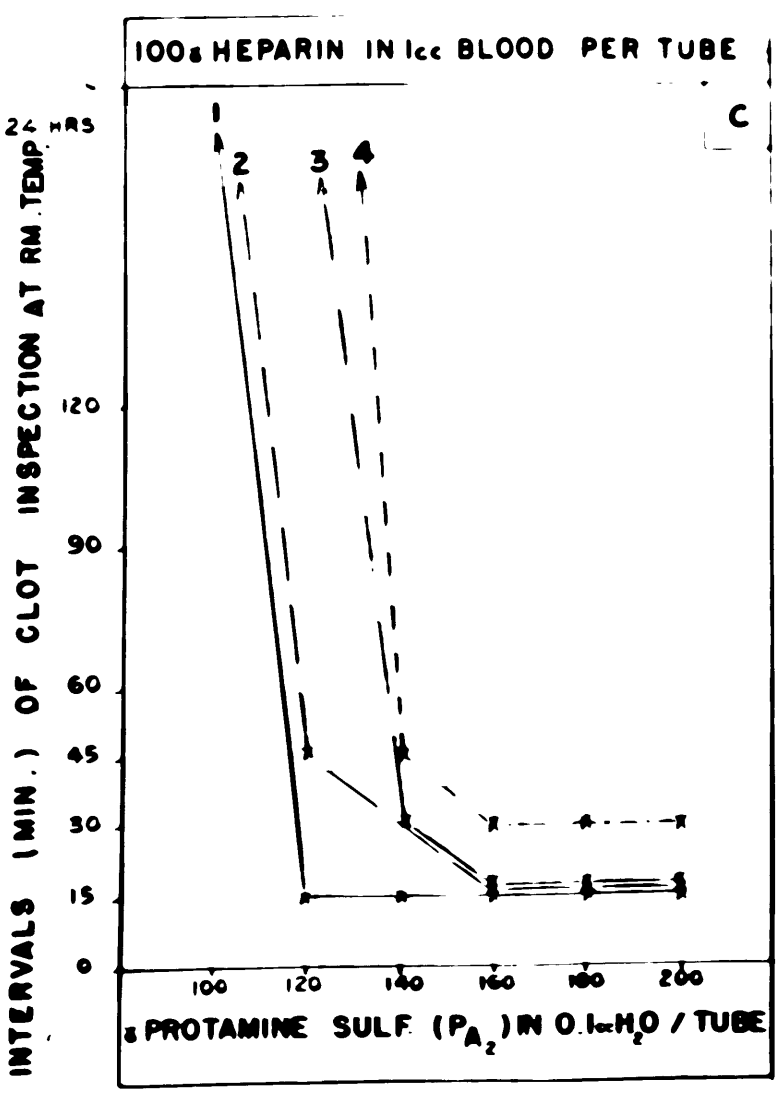


Fig.6.


100 γ HEPARIN IN 1cc BLOOD PER TUBE
A
24 HR
120
90
60
45
30
15
INTERVALS (MIN) OF CLOT INSPECTION AT RM TEMP
100 120 140 160 180 200
γ PROT SULF (P_{A_2}) IN 0.1cc H_2O / TUBE


100 γ HEPARIN IN 1cc BLOOD PER TUBE
B
120
90
60
45
30
15
INTERVALS (MIN) OF CLOT INSPECTION AT RM TEMP
100 120 140 160 180 200
γ PROT. SULF (P_{A_2}) IN 0.1cc H_2O PER TUBE


100 γ HEPARIN IN 1cc BLOOD PER TUBE
C
24 HRS
120
90
60
45
30
15
0
INTERVALS (MIN.) OF CLOT INSPECTION AT RM TEMP
100 120 140 160 180 200
γ PROTAMINE SULF (P_{A_2}) IN 0.1cc H_2O / TUBE

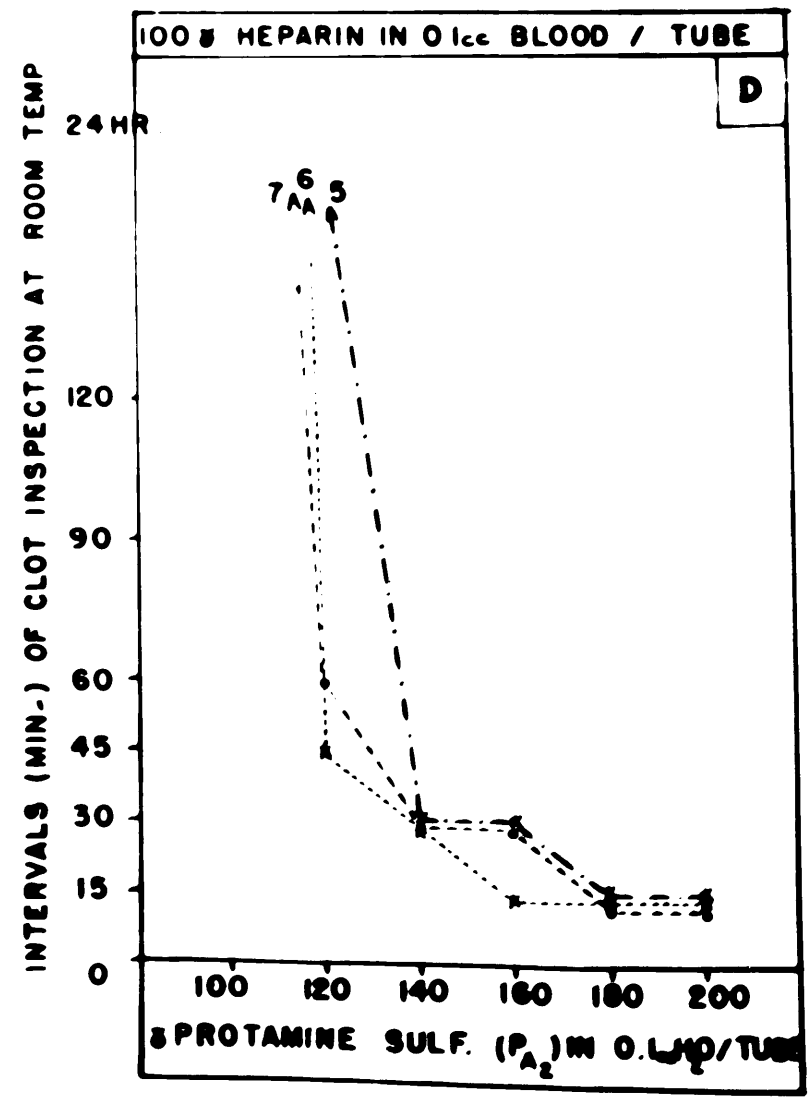
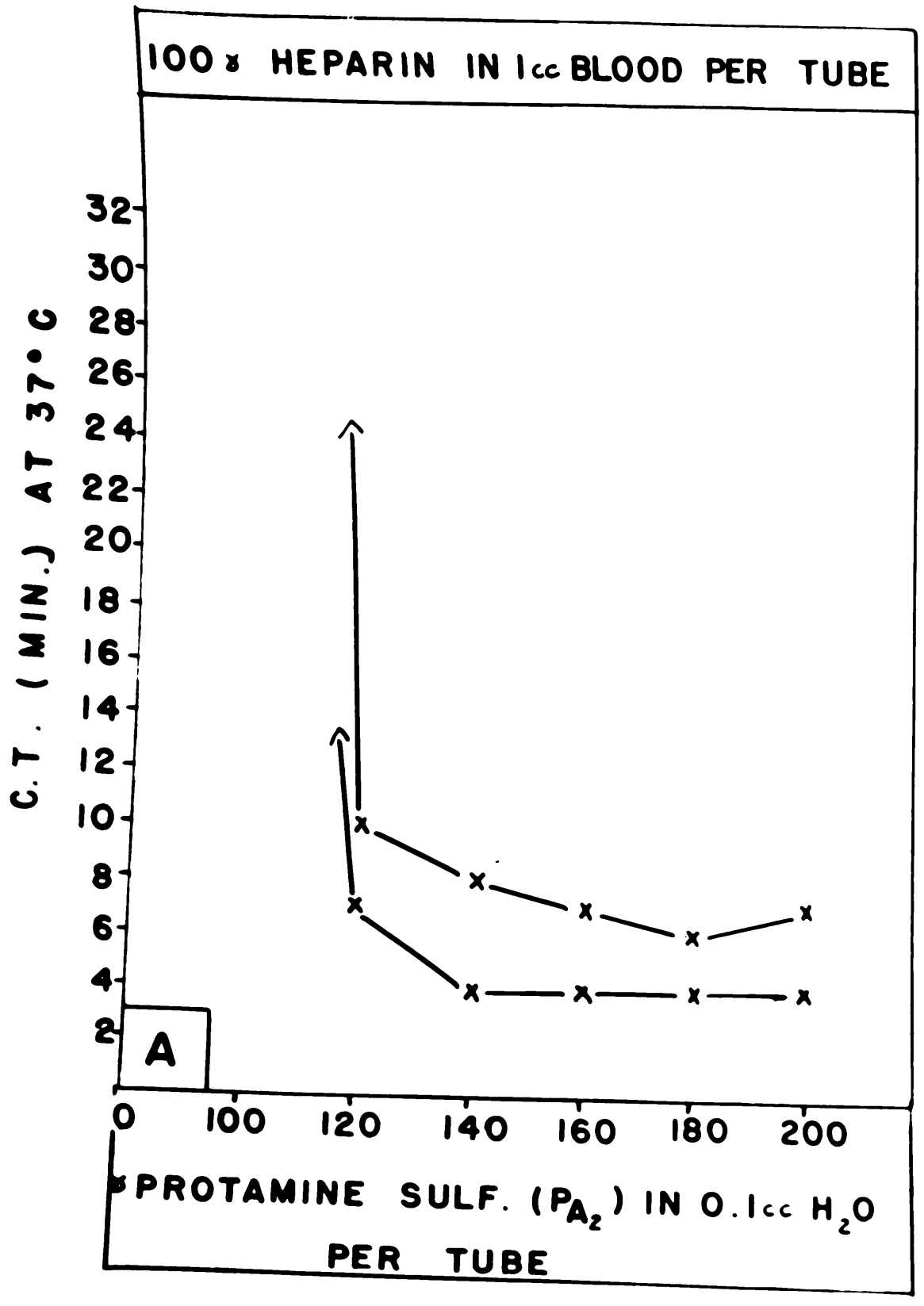

100 γ HEPARIN IN 0.1cc BLOOD / TUBE
D
24 HR
120
90
60
45
30
15
0
INTERVALS (MIN.) OF CLOT INSPECTION AT ROOM TEMP
100 120 140 160 180 200
γ PROTAMINE SULF. (P_{A_2}) IN 0.1cc H_2O/TUBE

Fig.7.


100 γ HEPARIN IN 1cc BLOOD PER TUBE
C.T. (MIN.) AT 37° C
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
A
0
100
120
140
160
180
200
γ PROTAMINE SULF. (P_{A_2}) IN 0.1cc H_2O PER TUBE

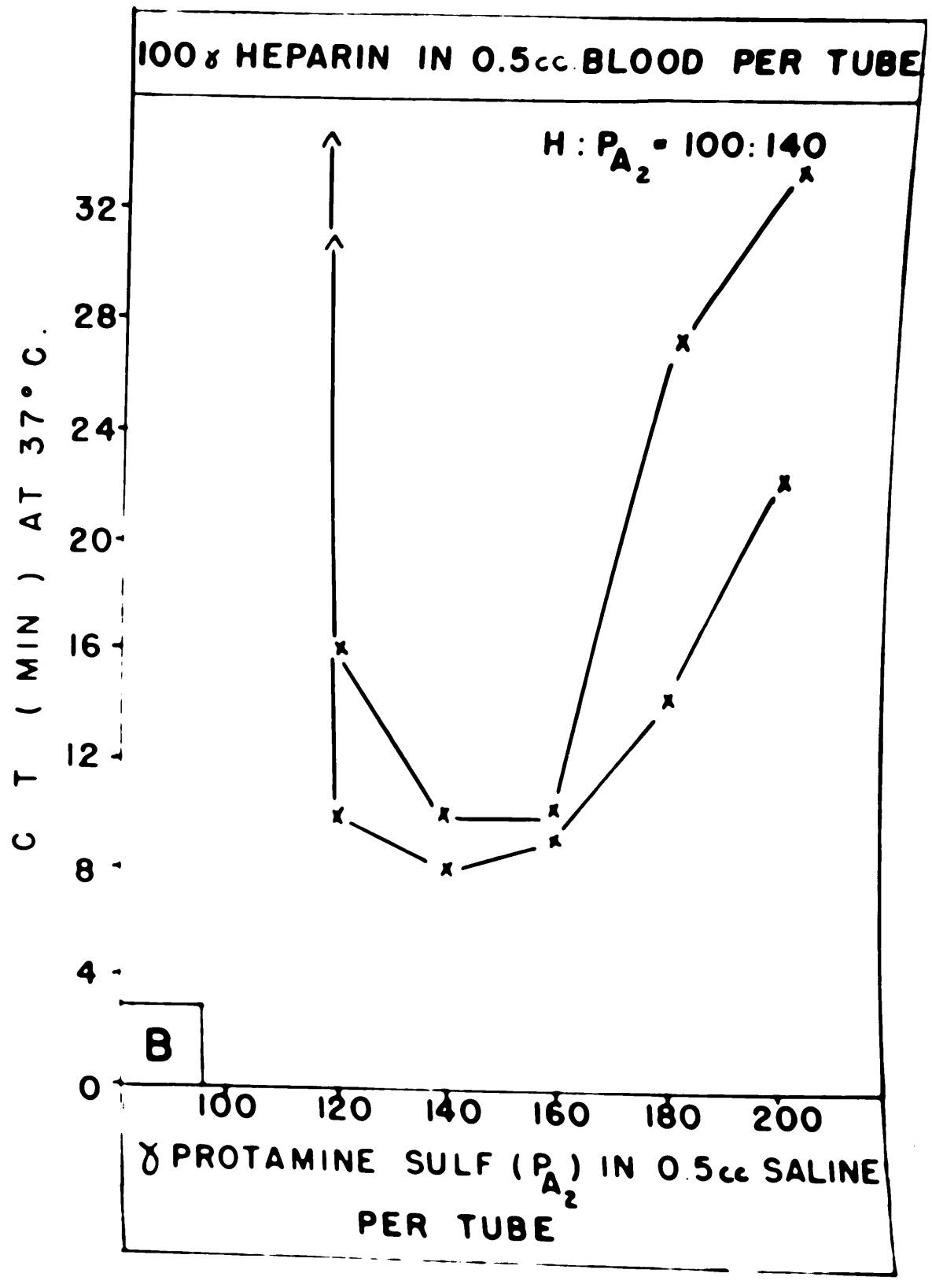
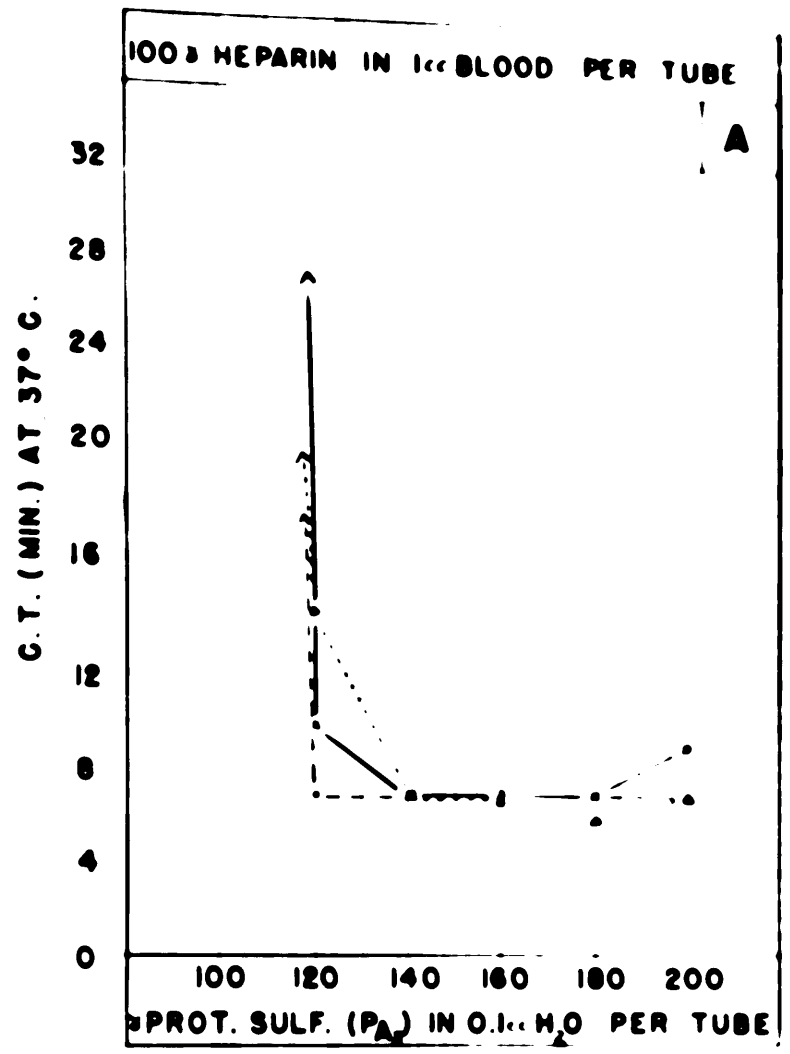
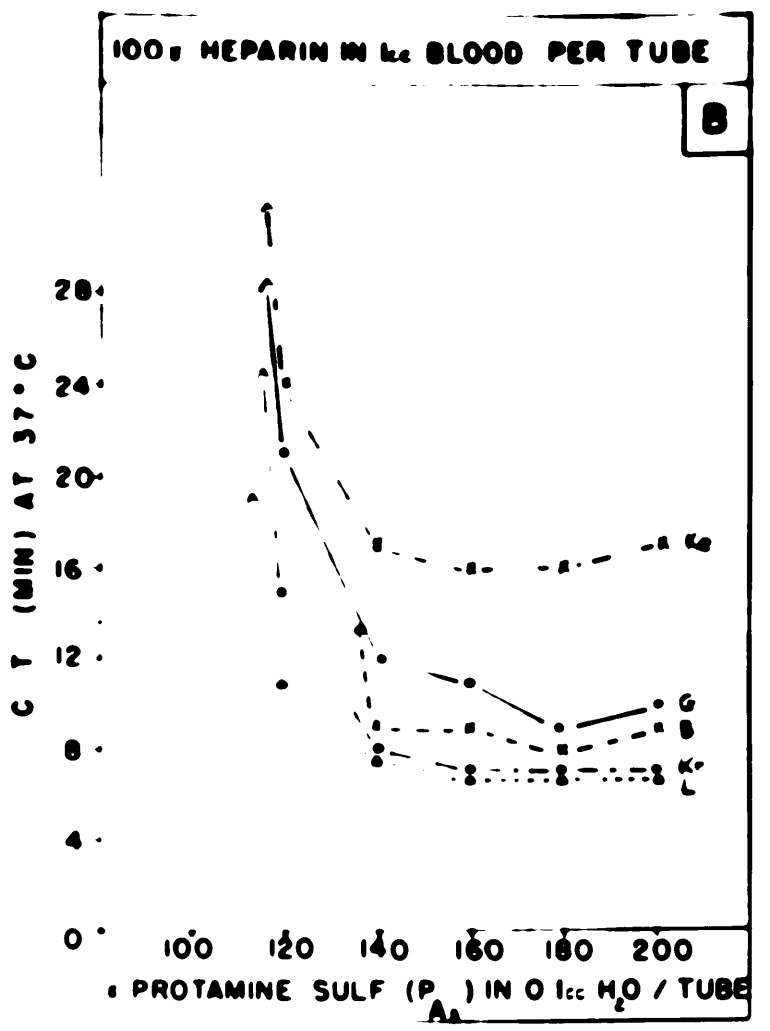
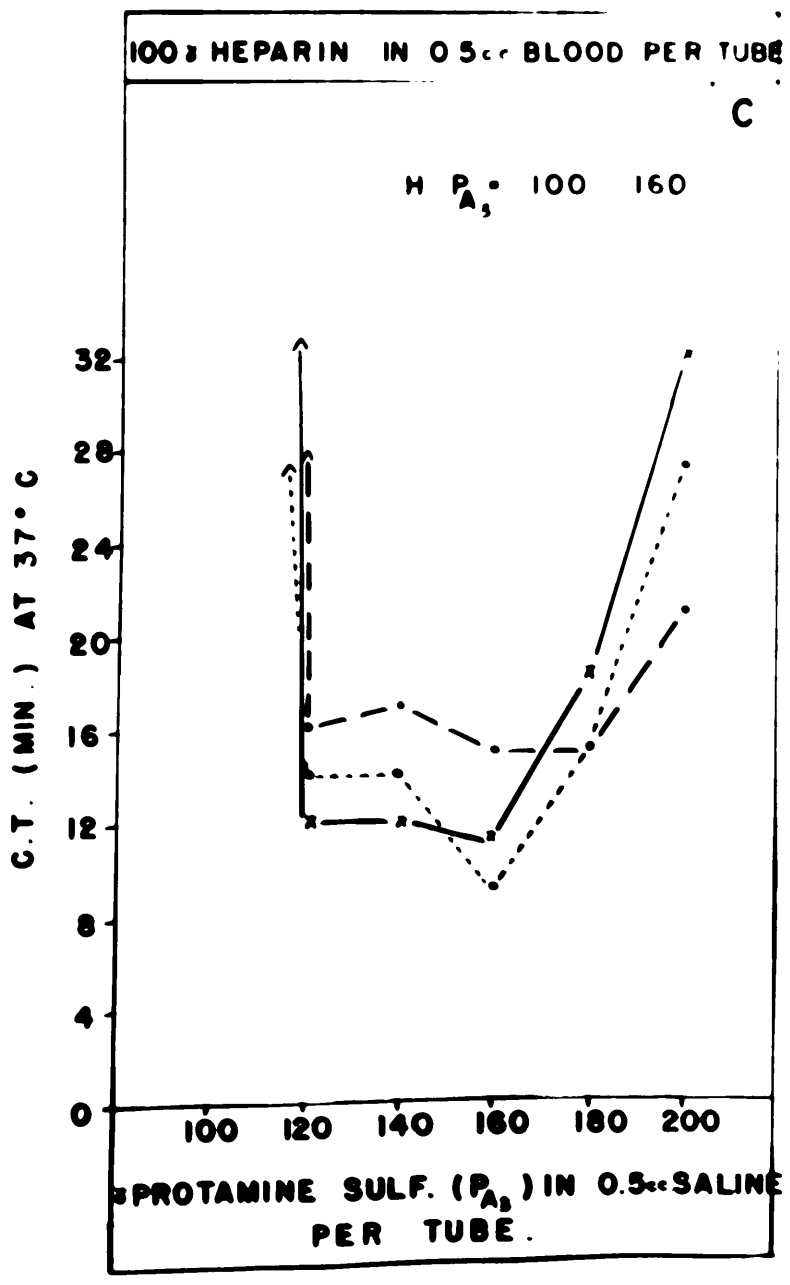

100 γ HEPARIN IN 0.5cc BLOOD PER TUBE
H : P_{A_2} = 100 : 140
C T (MIN.) AT 37° C.
32
28
24
20
16
12
8
4
B
0
100
120
140
160
180
200
γ PROTAMINE SULF (P_{A_2}) IN 0.5cc SALINE PER TUBE

Fig.8.


100 γ HEPARIN IN 1cc BLOOD PER TUBE
A
C.T. (MIN.) AT 37° C.
0
4
8
12
16
20
24
28
32
100
120
140
160
180
200
γ PROT. SULF. (P_As) IN 0.1cc H2O PER TUBE


100 γ HEPARIN IN 1cc BLOOD PER TUBE
B
C.T. (MIN) AT 37° C
0
4
8
12
16
20
24
28
100
120
140
160
180
200
γ PROTAMINE SULF (P_As) IN 0.1cc H2O / TUBE
G
B
Ke
L


100 γ HEPARIN IN 0.5cc BLOOD PER TUBE
C
H P_As = 100 160
C.T. (MIN.) AT 37° C
0
4
8
12
16
20
24
28
32
100
120
140
160
180
200
γ PROTAMINE SULF. (P_As) IN 0.5cc SALINE PER TUBE.

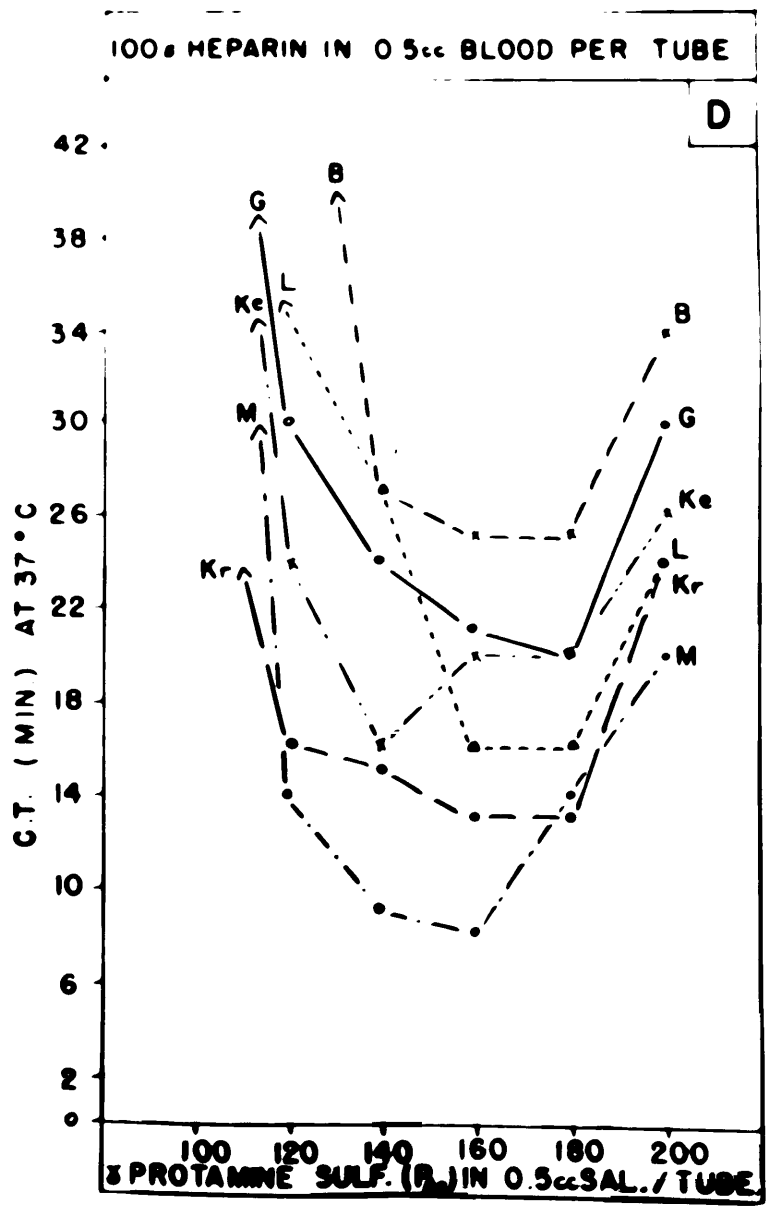
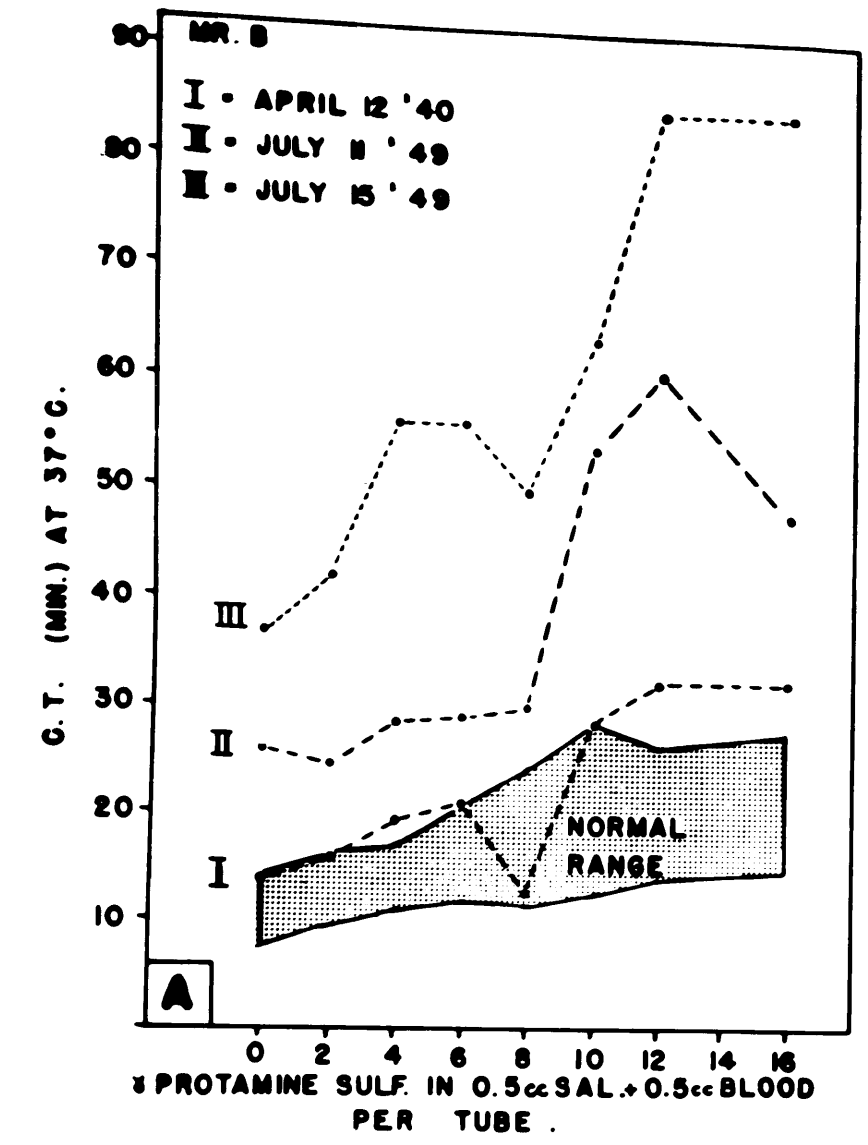
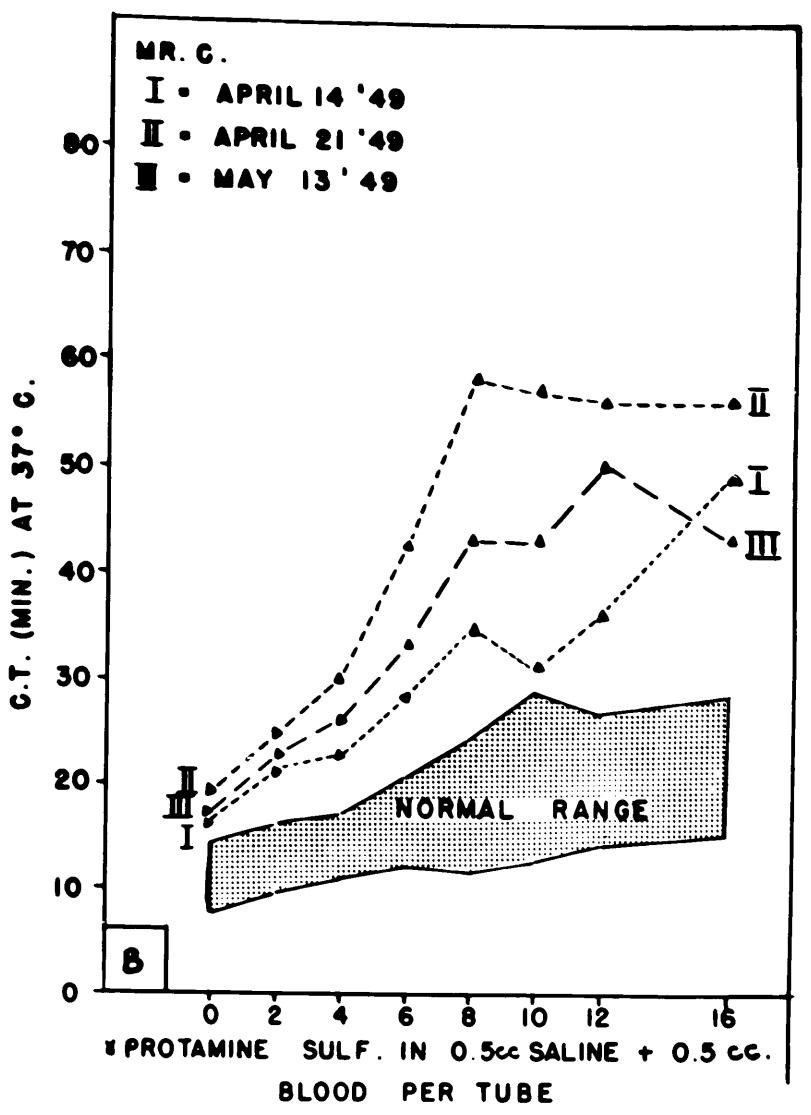
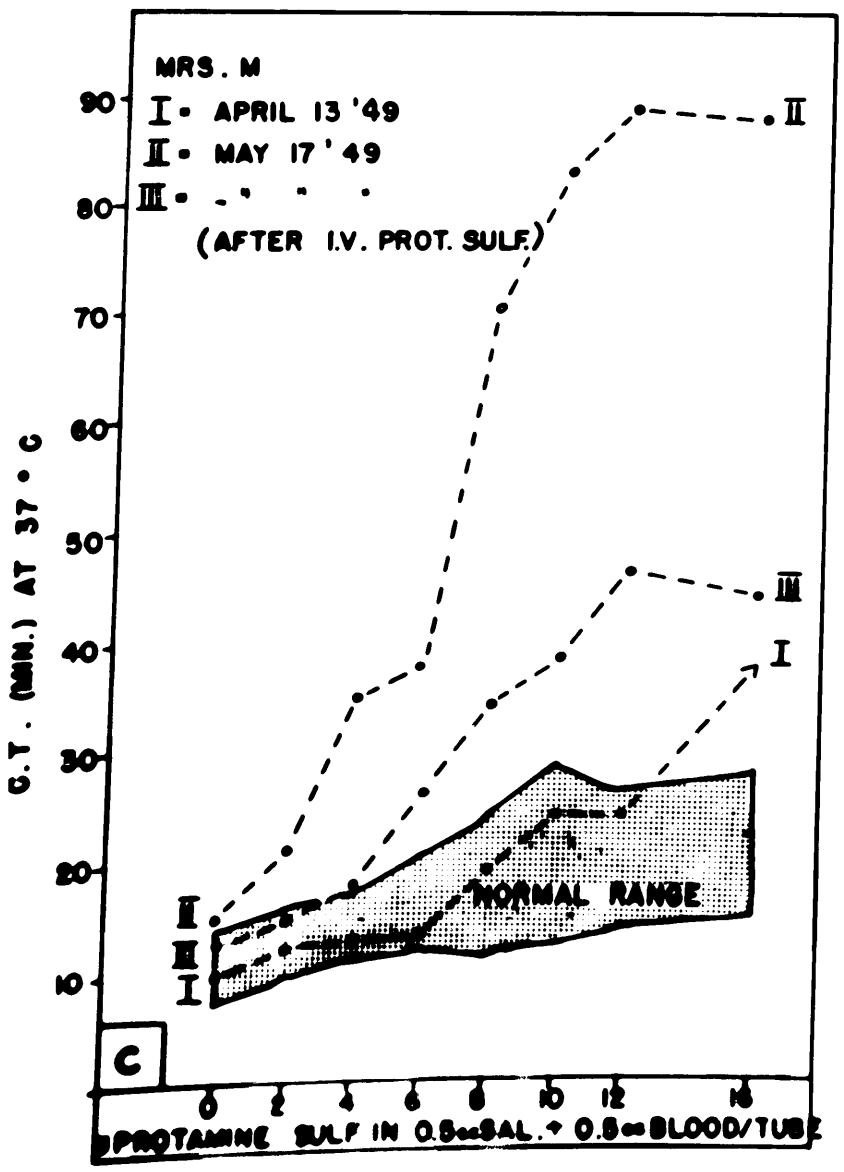

100 γ HEPARIN IN 0.5cc BLOOD PER TUBE
D
C.T. (MIN) AT 37° C
0
2
6
10
14
18
22
26
30
34
38
42
100
120
140
160
180
200
γ PROTAMINE SULF. (P_As) IN 0.5cc SAL. / TUBE
G
B
Ke
L
M
Kr

Fig.9.


MR. B
I • APRIL 12 '40
II • JULY 11 '49
III • JULY 15 '49
NORMAL RANGE
A
C.T. (MIN.) AT 37° C.
0 2 4 6 8 10 12 14 16
% PROTAMINE SULF. IN 0.5cc SAL. + 0.5cc BLOOD PER TUBE.


MR. C.
I • APRIL 14 '49
II • APRIL 21 '49
III • MAY 13 '49
NORMAL RANGE
B
C.T. (MIN.) AT 37° C.
0 2 4 6 8 10 12 16
% PROTAMINE SULF. IN 0.5cc SALINE + 0.5 cc. BLOOD PER TUBE


MRS. M
I • APRIL 13 '49
II • MAY 17 '49
III • - • • •
(AFTER I.V. PROT. SULF.)
NORMAL RANGE
C
C.T. (MIN.) AT 37° C
0 2 4 6 8 10 12 16
% PROTAMINE SULF IN 0.5cc SAL. + 0.5cc BLOOD/TUBE

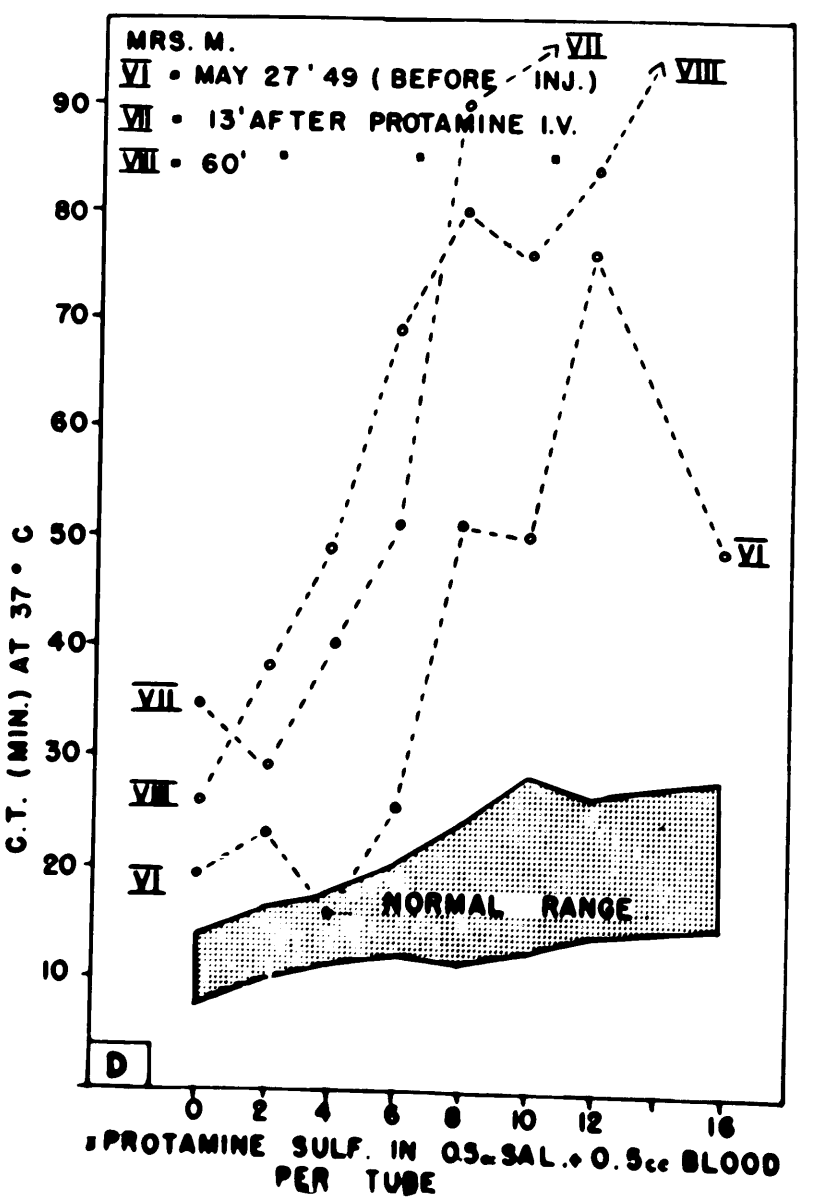

MRS. M.
VI • MAY 27 '49 (BEFORE INJ.)
VII • 13' AFTER PROTAMINE I.V.
VIII • 60' •
NORMAL RANGE
D
C.T. (MIN.) AT 37° C
0 2 4 6 8 10 12 16
% PROTAMINE SULF. IN 0.5cc SAL. + 0.5cc BLOOD PER TUBE

Fig.10.

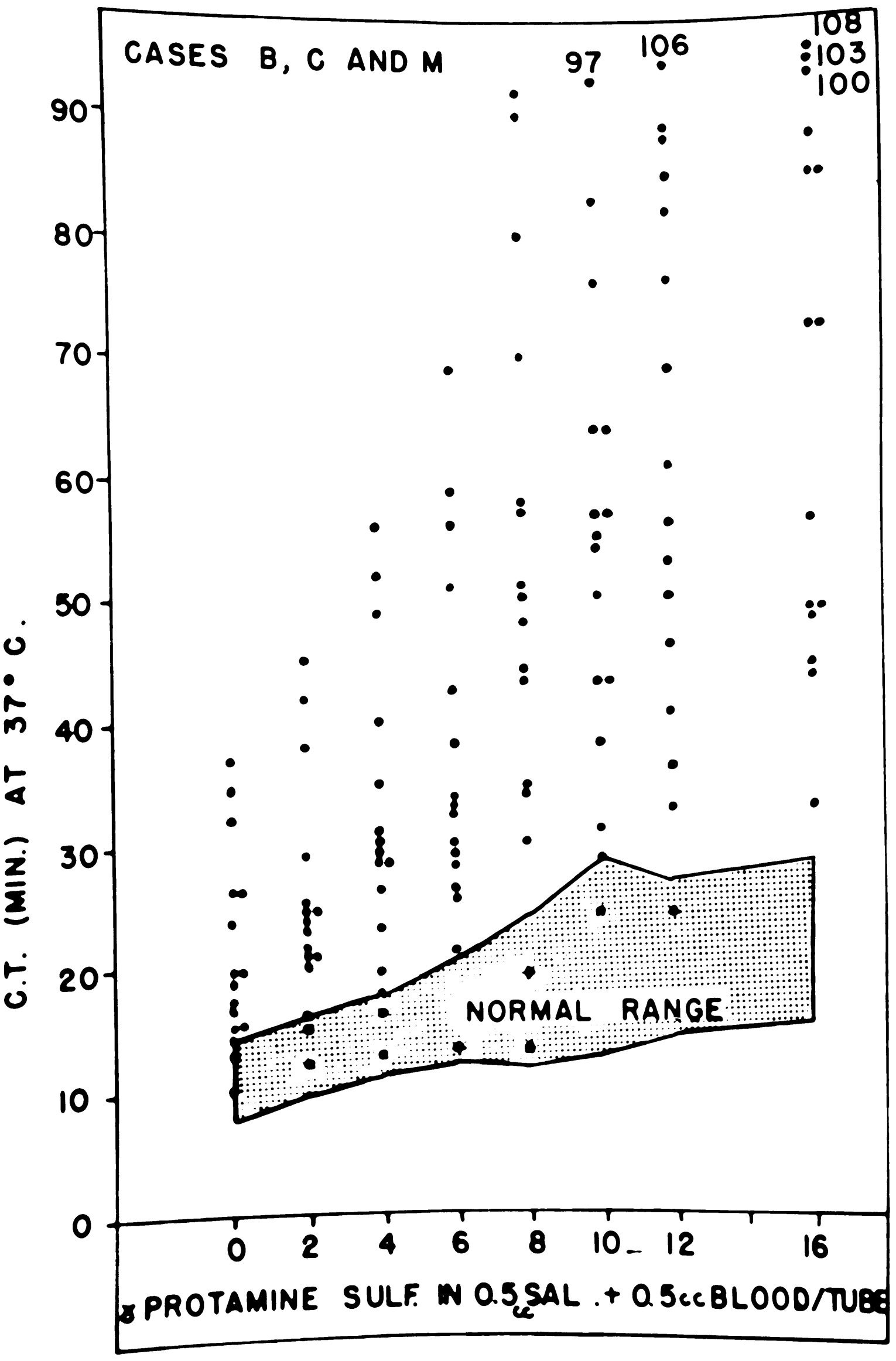
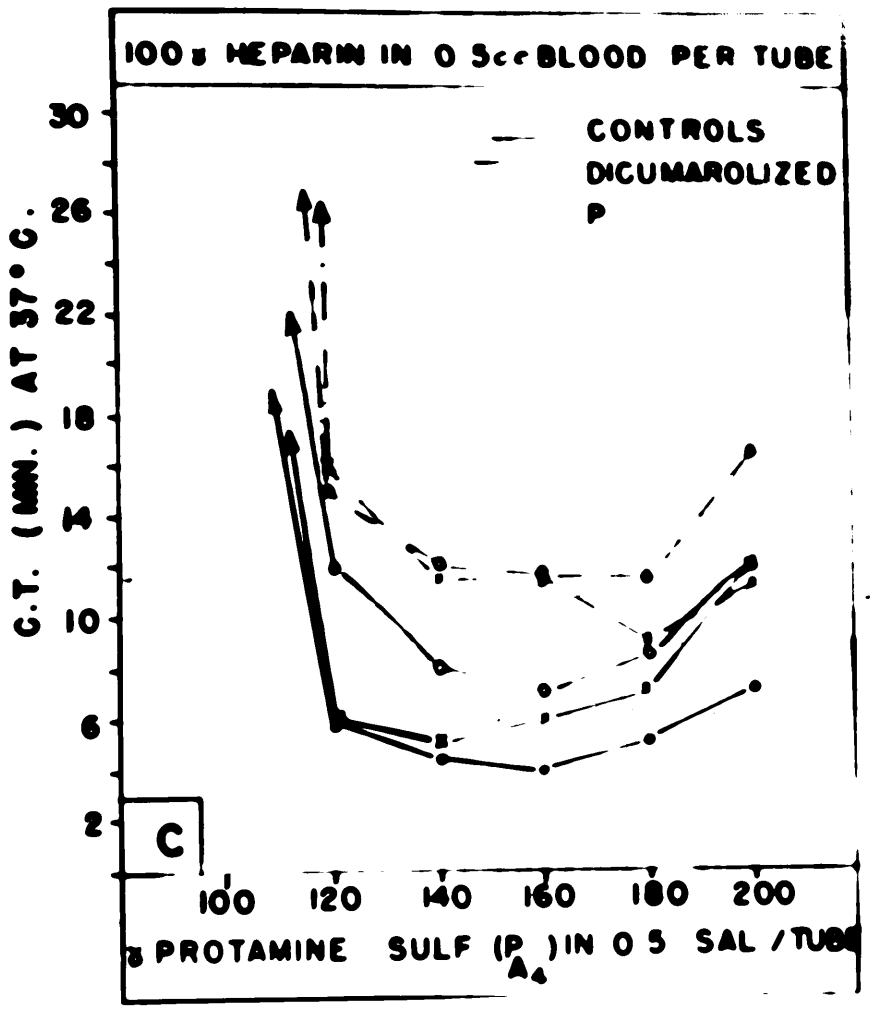
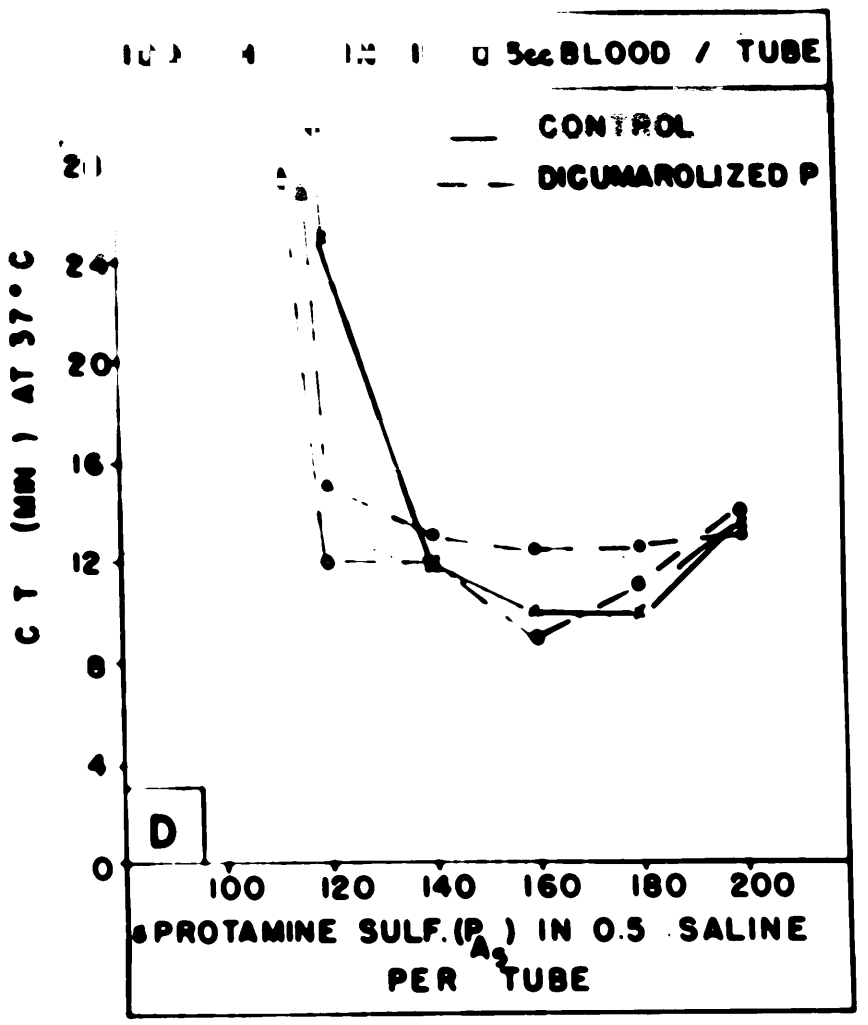
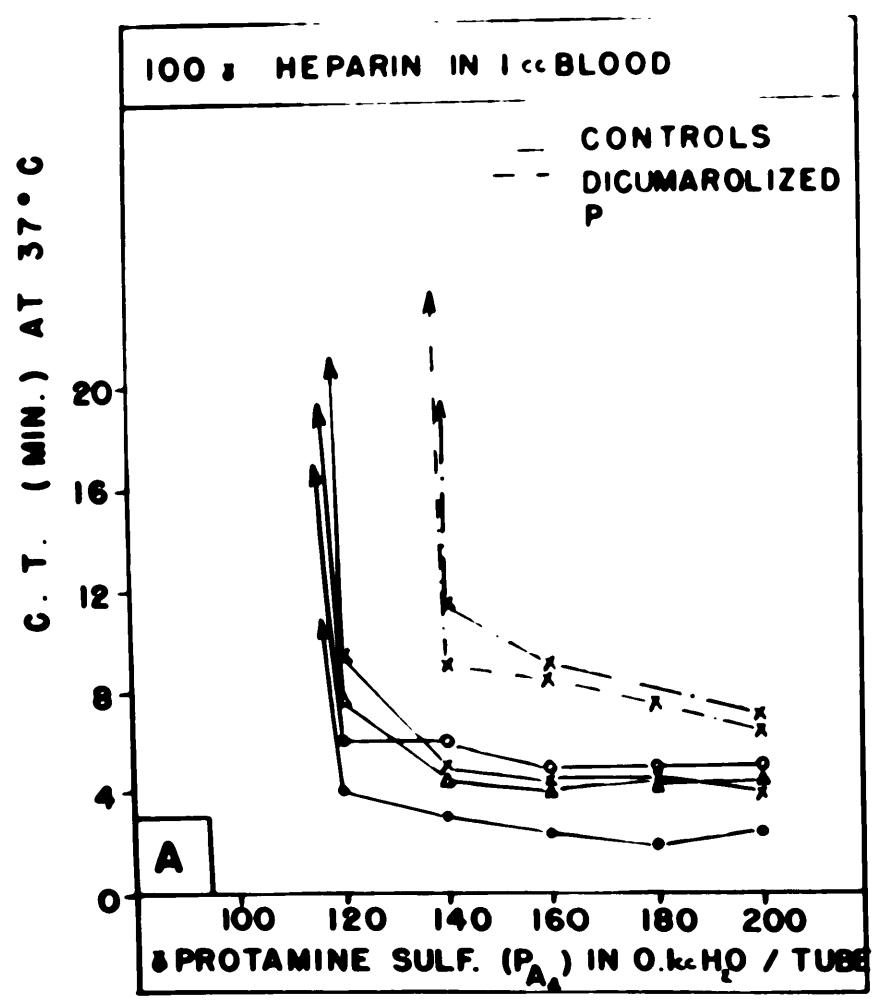
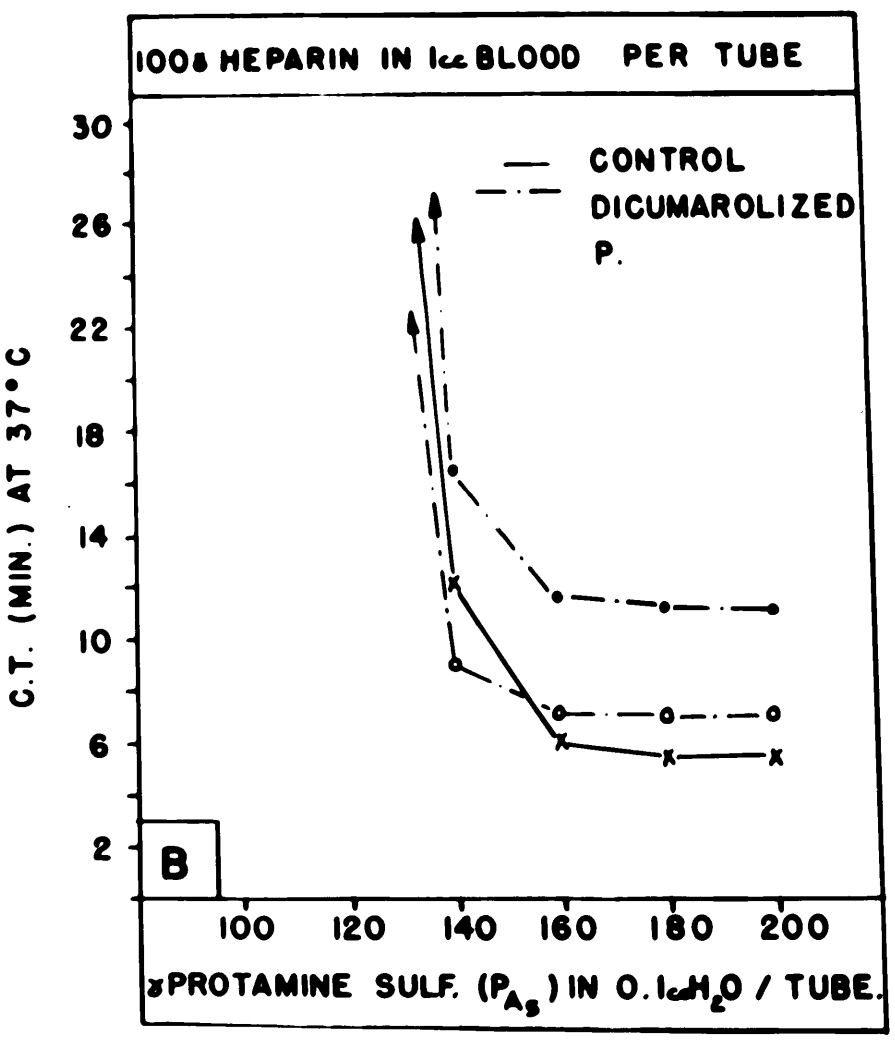

CASES B, C AND M
97
106
108
103
100
90
80
70
60
50
40
30
20
10
0
C.T. (MIN.) AT 37° C.
NORMAL RANGE
0
2
4
6
8
10
12
16
PROTAMINE SULF. IN 0.5 cc SAL. + 0.5 cc BLOOD/TUBE

Fig.11.


100 ϒ HEPARIN IN 0.5cc BLOOD PER TUBE
CONTROLS
DICUMAROLIZED P
C.T. (MIN.) AT 37° C.
30
26
22
18
14
10
6
2
C
100 120 140 160 180 200
ϒ PROTAMINE SULF (P A4) IN 0.5 SAL / TUBE


0.5cc BLOOD / TUBE
CONTROL
DICUMAROLIZED P
C T (MIN) AT 37° C
24
20
16
12
8
4
0
D
100 120 140 160 180 200
ϒ PROTAMINE SULF. (P A3) IN 0.5 SALINE PER TUBE


100 ϒ HEPARIN IN 1 cc BLOOD
CONTROLS
DICUMAROLIZED P
C. T. (MIN.) AT 37° C
20
16
12
8
4
0
A
100 120 140 160 180 200
ϒ PROTAMINE SULF. (P A4) IN 0.1cc H2O / TUBE


100ϒ HEPARIN IN 1cc BLOOD PER TUBE
CONTROL
DICUMAROLIZED P.
C.T. (MIN.) AT 37° C
30
26
22
18
14
10
6
2
B
100 120 140 160 180 200
ϒ PROTAMINE SULF. (P A3) IN 0.1cc H2O / TUBE.