STRESS-INDUCED CHANGES IN BLOOD COAGULATION IN THE RAT

by

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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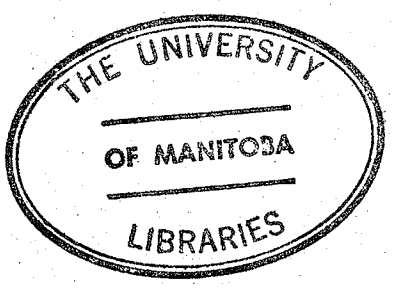

ABSTRACT

Present literature on the effects of stress on blood coagulability reveals a number of apparent inconsistencies. Although some observations have indicated stress-induced states of both hypercoagulability and hypocoagulability, others have found no evidence of change. To resolve existing discrepancies, improved techniques of measurement were employed which permitted the assessment of stress effects on specific components of the blood coagulation mechanism.

In a series of five experiments, the effects of various stressors on the blood coagulation system of the rat were investigated. Stress-induced changes were found in all five experiments. Animals subjected to the stress of restraint-cold showed evidence of a severe hypocoagulable state, characterized by prolongation in the partial thromboplastin time and lowered levels of Factor VIII. Thrombin times of stressed animals were significantly shorter than those of controls. Increasing durations of restraint-cold caused progressive alterations in coagulation parameters. Changes evident after only 10 minutes of restraint-cold stress became progressively greater over the intervals tested.

The results appeared to be inconsistent with the stress-hypercoagulability hypothesis of previous investigators. However, closer examination of the pattern of results suggested that the observed hypocoagulability was preceded by an earlier hypercoagulable state during which the coagulation mechanism was triggered, and coagulation occurred in vivo. The resulting hypocoagulability was attributed to the consumption and depletion of plasma clotting

factors as a result of intravascular coagulation.

Possible mechanisms of stress effects on blood coagulation were considered. It was suggested that stress-induced changes in coagulation may be mediated by the pituitary-adrenal axis and the elevation of circulating catecholamines. Examination of the psychological conditions during which such changes have resulted suggested that emotional states dominated by fear, anxiety or hostility may contribute to thrombosis.

ACKNOWLEDGEMENTS

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What is hardest of all to do?

What seems to you the easiest:

To see with your own eyes,

What your eyes lay before you.

Goethe

CHAPTER 1

INTRODUCTION

This study was initiated following the serendipitous observation that rats subjected to the dual stress of restraint-cold, as a means of inducing gastric ulceration, manifested a severe hemorrhagic tendency characterized by bleeding and petechiae. Such observations appeared to be inconsistent with previous studies which have reported increased coagulability (hypercoagulability) due to stress (Cannon & Grey, 1914; DeLong, Uhley & Friedman, 1959; Dreyfuss, 1956; Friedman & Uhley, 1959; Macht, 1952; Schneider & Zangari, 1952).

Critical examination of the literature revealed a number of major inconsistencies which appeared to be difficult to reconcile.

A number of investigators (Cannon and Gray, 1914; Dreyfuss, 1956; Macht, 1952, Schneider, 1951; Schneider and Zangari, 1952) reported increased blood coagulability as a result of emotional stress. Similar findings have been reported in animal experiments (DeLong, Uhley, and Friedman, 1959; Friedman and Uhley, 1959; Uhley and Friedman, 1959). However, other researchers (Jaques, 1964; Rozynkowa and Bielski, 1965; Ruxin, Bidder, and Agle, 1972) have found no evidence for the stress-hypercoagulability hypothesis.

Stress-induced increases in fibrinolysis have also been reported (Biggs, MacFarland and Pilling, 1947; Ogston, 1964; Ogston, McDonald and Fullerton, 1962; Truelove, 1951). However, other authors (Cash, 1972; Cash and Allan, 1967) have found decreased fibrinolysis in response to stress.

On the basis of these reports, the influence of stress on blood coagulation and fibrinolysis is a controversial issue. In order to evaluate the findings, these studies will now be critically reviewed.

Review of the Literature

(a) Stress and Blood Coagulation

Cannon (1914), in his classic work on the influence of major emotions on various bodily functions, reported a shortening of the clotting time in cats following exposure to stimuli causing pain, fear or rage. This acceleration of coagulation (hypercoagulability) was considered to be one of several adaptive responses designed to prepare the organism for "fight or flight". Thus, it was reasoned,in the case of physical injury the blood would clot rapidly, thereby preventing life threatening hemorrhage. The mechanism underlying this phenomenon was not known, but results were attributed to the effects of epinephrine initiated by sympathetic stimulation via the adrenal glands.

Researchers did not attempt to extend these observations until the 1950's. In a study of the effects of exercise, pain and emotional stress on various physiological functions, Schneider (1950) reported a reduction in the whole blood clotting time (accelerated coagulation) during a stressful interview situation. Schneider (1951) reported a shortening of whole blood clotting time (WBCT), increased blood viscosity and elevated blood pressure during periods of anxiety, fear, anger or hostility. In contrast, the clotting time was consistently prolonged during states of depression. These reports of stress-induced hypercoagulability were based on observations of

six individuals suffering from thrombophebitis. However, in the same study four healthy subjects examined under comparable periods showed no evidence of accelerated coagulation. In a further study, Schneider and Zangari (1951) reported similar findings, although stress-induced hypercoagulability was found only during periods of stress-induced blood pressure elevation.

Macht (1952) reported results which tended to suggest the existence of a direct relationship between the intensity of emotion and speed of blood coagulation. In this study blood donors were divided into three groups (anxious, apprehensive, or calm) on the basis of the authors opinion. Clotting times of the calm group were found to range from 8-12 minutes, the apprehensive group, 4-5 minutes, and the highly anxious group, 1-3 minutes. This study may, however, be criticized since no behavioural criteria for the assessment of anxiety were specified. Subjects were assigned to each group simply on the basis of vague subjective criteria of the investigator. In addition, no description of the size of each group nor details of the procedure were included.

Dreyfuss (1956) sought to further explore the relationship between emotional stress and hypercoagulability in 36 healthy volunteers under the stress of final examinations. Accelerated coagulation was observed in 7 subjects using the glass WBCT and in 22 subjects using siliconized tubes. Statistical evaluation of the results showed average change of 1.23 and 5.1 minutes for glass and silicone respectively - values that are within the range of error of the technique. Although the study appears to support the stress-hypercoagulability hypothesis, it should be emphasized that 14

subjects showed either no changes in coagulation, or changes in the opposite direction.

Subsequent investigators (Friedman and Rosenman, 1959; Friedman, Rosenman and Carroll, 1958) have studied the coagulation mechanism and its relation to a coronary-prone behaviour pattern. Individuals exhibiting a specific dominant overt pattern of behaviour characterized by competitive drive, aggressiveness, feelings of being under time pressures (Pattern A), showed accelerated clotting times during periods of occupational stress, in comparison to subjects exhibiting other behaviour patterns. These studies, however, did not include control groups, analysis of diet, medication or physical activity, all of which may conceivably affect the results.

In a series of studies(DeLong, Uhley and Friedman, 1959; Friedman and Uhley, 1959; Uhley and Friedman, 1959)examined the effects of acute and chronic environmental stress on blood coagulability. Rats were exposed to avoidable shock, by periodic electrical changing of alternate halves of a grid floor. This situation evoked responses of fear and apprehension. Exposure to this form of stress for periods of one, three, or six hours, caused an acceleration of WBCT (DeLong, Uhley and Friedman, 1959). Hypercoagulability was also found in adrenalectomized animals similarly stressed (Friedman and Uhley, 1959). Thus the initial hypothesis attributing hypercoagulability to increased levels of epinephrine (Cannon and Mendenhall, 1914) was not supported.

In an attempt to determine whether chronic exposure to the same stress would lead to similar changes, a group of animals were subjected to the same environmental conditions for a period of ten months (Uhley and Friedman, 1959). During this time both experimental

and control animals were maintained on a diet high in fat and cholesterol. Results demonstrated a reduction in WBCT, and higher lipid and cholesterol levels in the stressed group of rats. However, investigators have subsequently reported hypercoagulability, induced by diets high in fats and cholesterol (Davidson, Howard and Gresham,, 1962; Renaud and Lecompte, 1970). Thus, the results of this particular study are equivocal at best. A further problem, common to this series of studies, is the use of capillary blood in determining WBCT. Samples obtained by this method are invariably contaminated by tissue extracts, yielding results which are of questionable reliability or validity.

Other experiments have been concerned with changes in blood clotting in neurotic or psychotic states (Aleksandrowicz, Dzikowski and Schiffer, 1964; Kast and Zweibel, 1954; Vuori, 1950). Results of these studies are difficult to interpret, since adequate controls for diet, medication, activity or other variables were not included. In most cases sample size has been too small to allow for meaningful generalizations.

Kast and Zweibel (1954) reported progressively decreasing clotting times in schizophrenics prior to electroshock therapy. Such findings were attributed to fear or anxiety over the impending shock. In addition to problems previously mentioned, these authors used the Detakats (1950) modification of the WBCT, involving the addition of heparin to the blood sample. However, since the anti-coagulant effect of heparin is inversely proportional to platelet concentration (Conley, Hartmann and Lalley, 1948) and, since an increase in platelets due to stress has been reported (Cash, 1972),

the accelerated clotting times may have been erroneously interpreted.

Aleksandrowicz et al. (1964) on the basis of thrombo-elastographic findings, concluded that neither psychotic nor neurotic processes lead to changes in coagulation. Changes were observed only in conjunction with psychomotor excitement.

The studies discussed to date have been cited by researchers as providing conclusive evidence for a positive relationship between stress and hypercoagulability. In a majority of cases these reports are based on evidence obtained from the WBCT, and in many cases the observed mean differences lie well within the standard error of the technique. Closer examination of these studies indicates that the evidence for the stress-hypercoagulability hypothesis is far from conclusive.

Results from a number of studies (Jaques, 1962; Jaques and Chubaty, 1954; Mogenson and Jaques, 1957; Rozynkowa and Bielski, 1965; Ruxin, Bidden and Agle, 1972) fail to support the stress-hypercoagulability hypothesis. Jaques and his associates have reported that under some conditions stress may act as a hemorrhagic agent. Diverse stressors such as restraint, insulin, conditioned fear, and electroshock resulted in a high incidence of spontaneous hemorrhage in dicumarolized rats. This hemorrhagic tendency was found to be unrelated to increased fibrinolytic activity (Lucas and Jaques, 1964) since administration of epsilonaminocaproic acid (EACA) - a competitive inhibitor of plasminogen activator, did not prevent hemorrhage. The Jaques et al. group of studies present problems in interpretation since coumadin anticoagulants were administered to "Make these procedures more effective" (Mogenson

and Jaques, 1957). However, coumarin compounds have varying speeds of absorption and metabolic degradation (Goth, 1972), thus a critical evaluation of these studies is virtually impossible.

Ruxin et al. 1972, using a controlled research design examined the influence of arousal on three indices of adrenergic activity - one of which included blood coagulability. Measures taken 5 minutes before and after electroshock therapy were compared with those taken several days later. No significant changes in WBCT and no correlation between coagulability and arousal were found.

Rozynkowa & Bielski (1965) in a thromboelastographic study, reported decreased clot elasticity and, prolonged time of growth of the clot in rats subjected to avoidable shock. Such animals also showed increased variability in clot elasticity. In addition, a single presentation of electroshock was sufficient to cause increased variability in clot elasticity parameters.

These results are however somewhat equivocal, since blood samples were drawn before and after stress. Although the intra-subject differences were compared with those of a control group in which sampling was performed on two separate occasions, the possibility remained that the stress treatment may have interacted with the stress of the sampling procedure. Thus, the stress of blood sampling may not have been adequately controlled between groups.

In a series of studies designed to evaluate the influence of diverse stressors on blood fibrinogen level, Hardaway, Johnson, Houchin, Jenkins, Burns & Jackson (1964) reported stress-induced elevations in fibrinogen in dogs subjected to trauma, anaethesia

and anxiety. Such findings are consistent with a recent report of Chernigovskaya, Cherkovich & Uzunyan (1973). These authors found that prolonged emotional stress resulted in a marked increase in fibrinogen level of healthy monkeys. In this study, stress was induced by disruption of the hierarchical relationships within the group, and by disturbing the normal routine of day-light cycle and feeding periods. Although a variety of coagulation indices were studied, (prothrombin time, recalcification time, platelet adhesiveness) no significant changes were found during the period of stress.

The influence of the autonomic nervous system on the coagulation mechanism now appears to be well documented. Various authors (Gunn & Hampton, 1967; Kubantseva, 1973; Zubiarov, 1966) have found that the hypothalamus, the highest autonomic center, has an influence on the coagulation mechanism. Gunn & Hampton (1967) localized hypothalamic and mesencephalic nuclei in the regulation of Factor VIII levels in dogs. Stimulation of areas of the hypothalamus and reticular formation resulted in increased levels of Factor VIII, whereas decreased levels were related to stimulation of the hippocampus and mamillary nuclei.

Other investigators (Markosyan & Yakunin, 1962) have shown that stimulation of the anterior hypothalamus gives rise to hypocoagulability whereas stimulation of the posterior hypothalamus produced hypercoagulation.

This line of research is now being actively pursued by Russian investigators. In an attempt to resolve some existing controversies, Kubantseva (1973) studied the effect of stimulating the anterior and posterior zones of the hypothalamus at different

frequencies. Stimulation of the posterior hypothalamus resulted in hypercoagulation, regardless of frequency. However, the effects of stimulating the anterior hypothalamus were dependent on frequency employed.

Despite this most recent line of investigation, research on the effects of stress on blood coagulation is characterized by major discrepancies. These discrepancies are difficult to resolve, because in studies of this type a great number of variables must be strictly controlled, and problems of research design carefully considered, in order to obtain reliable and meaningful data.

The studies previously discussed illustrate the paucity of research on the effects of stress on the coagulation mechanism. The few available reports indicate inconsistent or ambiguous findings. In general, the majority of studies have failed to include control groups (Cannon & Gray, 1914; Dreyfuss, 1956; Friedman & Rosenman, 1959; Friedman et al, 1958; Macht, 1952; Schneider & Zangari, 1951), standardization of diet (Dreyfuss, 1956; Friedman et al, 1958; Friedman & Rosenman, 1959; Uhley & Friedman, 1959), control of extraneous variables such as drugs (Aleksandrowicz et al, 1964; Friedman et al, 1958; Kast & Zweibel, 1954; Schneider & Zangari, 1951) or activity (Aleksandrowicz et al, 1964; DeLong et al, 1959; Friedman et al, 1958; Kast & Zweibel, 1954; Rozynkowa et al, 1965; Vuori, 1950). Reported mean differences in many cases have been within the standard error of the technique (Dreyfuss, 1956; DeLong et al, 1959; Friedman & Rosenman, 1959; Schneider & Zangari, 1951) and have been interpreted as indicants of meaningful functional alterations. Tests used to assess the

coagulation mechanism have in general been crude, unsophisticated, relatively inaccurate and subject to interpretation bias (Cannon & Mendenhall, 1914; DeLong et al, 1959; Friedman et al, 1958; 1959; Kast & Zweibel, 1954; Macht, 1952; Schneider, 1950; 1951; Schneider & Zangari, 1951; Uhley & Friedman, 1959; Vuori, 1950).

Results of these studies have been interpreted as providing conclusive evidence that stress is accompanied by increases in blood coagulability, and have been cited in reviews of psychosomatic interrelationships (Lachman, 1972). However, as this discussion has indicated, such evidence is far from convincing.

(b) Stress and Fibrinolysis

The observation of post mortem incoagulability following sudden violent death was probably the first indication of increased fibrinolysis following stress (Morawitz, 1906). Morawitz observed that blood from such cases contained no fibrinogen, and could destroy the fibrinogen and fibrin of normal blood. This prompted further investigations of the effects of less severe forms of stress, such as emotional stress induced by preoperative anxiety (MacFarlane and Biggs, 1946; MacFarlane, Biggs and Pilling, 1947), threat of venepuncture (Ogston, 1964; Ogston, McDonald and Fullerton, 1962), oral examinations (Sawyer, Fletcher, Alkgaersig and Sherry, 1960; Sherry, Lindemeyer, Fletcher and Alkjaersig, 1959; Truelove, 1951), or electroconvulsive shock (Fantl and Simon, 1948; Tyminski, Czestochowska and Szlabowicz, 1970).

MacFarlane and Biggs (1946) in a series of experiments found a high incidence of fibrinolysis in patients undergoing surgical procedures. Analysis of various factors such as the

effect of premedication, anaesthetic, and the surgical procedure itself, showed a considerable proportion of patients (approximately 50%) exhibited increased fibrinolysis during the preoperative, premedication period. In the absence of any other obvious explanation, the authors attributed the increased fibrinolysis to preoperative anxiety or fear. This conclusion was supported by independent observations of Latner (1947) who found increased fibrinolysis in cases of anxiety states following the stress of air raids.

Increased fibrinolytic activity has also been induced by a variety of stressful situations. These have included severe physical exercise (Biggs, MacFarlane and Pilling, 1947; Iatrides and Ferguson, 1963; Menon, Burke and Dewar, 1967; Sawyer et al., 1960, Sherry et al, 1959), electroconvulsive shock therapy (Fantl and Simon, 1948), hypnotic-induced anxiety (Truelove, 1951) and injections of adrenaline (Biggs et al., 1947; Genton, Kern and von Kaulla, 1961; Truelove, 1951). A common factor linking all stressful situations is that of systemic catecholamine release (Elmadgian, Hope and Lamson, 1957). Thus, Biggs et al. (1947) conclusion that the increased fibrinolytic response constitutes a component of the alarm reaction as defined by Selye (1946) appears justifiable. Truelove (1951) in a series of experiments observed increased fibrinolysis without accompanying adrenaline effects. Using delayed eosinopenia as an index of circulating adrenaline, Truelove found increased fibrinolysis in the absence of eosinopenia. The author did not, however, acknowledge the limitations of the eosinophil count as an index of catecholamine release.

Ogston, McDonald and Fullerton (1962) reported increased fibrinolytic activity and decreased plasma recalcification times in "anxious" subjects. In this study anxiety was induced by the threat of future venepuncture, and subjects were classified as anxious on the basis of criteria such as "agitation, tenseness, and verbal content". These findings were confirmed in a subsequent study (Ogston, 1964). Although the design and methods employed in both studies were superior to the majority of studies in this area, the assessment of anxiety was far from satisfactory. No assessment of inter-observer relability was made, thus errors in assigning subjects to anxious or non-anxious groups may have occurred.

Although the importance of emotional stress in stimulating fibrinolytic activity is supported by the studies previously discussed, other investigators (Sawyer et al, 1960; Sherry et al, 1959) have reported that not all emotional stress is accompanied by increased fibrinolysis. Prolonged mental stress has been shown to cause a depression in the fibrinolytic response to exercise although resting levels of plasminogen activator remained unaltered (Cash and Allan, 1967). In a recent publication, Cash (1972) concluded that environmental factors may have profoundly depressive effects on the fibrinolytic response to exercise. These apparent inconsistencies are difficult to reconcile, however, a number of explanations are possible. The most obvious concerns the duration of stress. Effects of acute short term stress may differ somewhat from those of chronic stress. Another possible explanation is based on the problem of variable reactivity between individuals.

Individuals may show marked variations in catecholamine levels in response to stress, particularly in the ratios of adrenaline and noradrenaline released. As previously mentioned, adrenaline evokes fibrinolytic activity (Biggs et al, 1947; Genton et al, 1961; Tanser and Smellie, 1964). However, noradrenaline evokes weak fibrinolytic activity (Cash, 1972) and in some cases fails to initiate a fibrinolytic response (Ishioka, Matsumura and Shimamoto, 1970). Thus, individuals who respond to stress by releasing small amounts of catecholamines, or with a dominant noradrenaline response, may exhibit no increase in fibrinolytic activity. However, this explanation is not completely satisfactory in explaining the results, since these studies were conducted on groups of individuals.

The studies previously discussed illustrate the lack of adequate research on the effects of stress on the fibrinolytic and hemostatic mechanisms. As previously indicated, the majority of findings are somewhat equivocal, due to methodological and conceptual limitations. However, during the 1950's when most of this research was conducted, investigators were severely limited by the techniques then available. Since that time coagulation research has been growing exponentially, resulting in the development of sensitive reliable tests which may be used to assess selected components of the coagulation mechanism.

In order to facilitate further discussion, a basic understanding of the hemostatic and fibrinolytic mechanisms and the theoretical basis underlying some of the tests which assess their function is essential.

The Blood Coagulation Mechanism

Contemporary theories of blood coagulation are based on the classical four-factor theory proposed by Morawitz (1905). Morawitz postulated that prothrombin is converted to thrombin by tissue thrombokinase (thromboplastin) and calcium ions. The thrombin thus formed results in the conversion of soluble fibrinogen to insoluble fibrin.

Modern multiple-factor theories of coagulation, although of considerably greater complexity, are basically an extension of the classical four-factor theory. The schema of coagulation based on the "cascade" (MacFarlane, 1964) or "waterfall" (Davie and Ratnoff, 1964) concepts depicts a series of proenzyme-enzyme transformations in which each enzyme activates the next proenzyme, until the final substrate-fibrinogen is reached. The theories of MacFarlane, Davie and Ratnoff, although independently conceived, are essentially similar. Despite occasional gaps and difficulties, these theories offer opportunities for experimental testing and provide an adequate framework for experimental design.

Main Stages of Coagulation

Blood coagulation may be considered to occur in three main stages.

Stage one - Development of thromboplastin activity as a result of the coagulation factors in the blood (the intrinsic system), and the admixtures of tissue factors (the extrinsic system).

Stage two - Conversion of prothrombin to thrombin.

Stage three - Conversion of fibrinogen to fibrin.

The interaction of these three main stages is schematized in Figure I.

Stage One - Formation of Thromboplastin

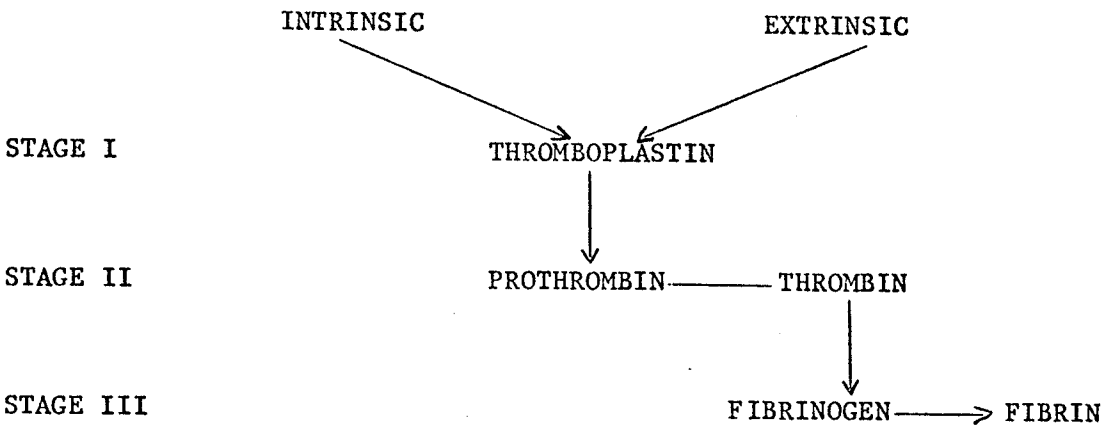
(a) The intrinsic system. The intrinsic mechanism is considered to function as a biological amplifier. Thus, at each level progressively greater amounts of proenzyme are generated resulting in continuous overall gain (MacFarlane, 1964).

The system is activated by contact of the blood with "foreign" surfaces or tissues other than normal vascular endothelium, thus factor XII is rendered enzymatically active (Ratnoff and Rosenblum, 1958). Activated factor XII acts on factor XI (Ratnoff, Davie and Mallett, 1961) which in turn activates factor IX (Ratnoff and Davie, 1962). In the next step, factor IXa alters factor VIII in such a way that it (VIIIa) converts factor X to its activated form (Xa). For this reaction to occur, calcium ions and phospholipids are necessary. The phospholipids needed at this step and further steps is furnished by the plasma and by the blood platelets. The reaction of factor Xa with factor V is complex and not fully understood. However, researchers believe that either an activated form of factor V or that a complex of factors Xa, V calcium and phospholipid is formed, resulting in the formation of thromboplastin (Biggs, 1972).

(b) The extrinsic system. This system is activated when the blood contacts tissue extracts. Tissue factors react with factor VII and calcium ions to form a complex which activates factor X. Once factor Xa has been formed, the extrinsic system

FIGURE 1

Interaction of the three main stages of coagulation.


INTRINSIC
EXTRINSIC
STAGE I
THROMBOPLASTIN
STAGE II
PROTHROMBIN
THROMBIN
STAGE III
FIBRINOGEN
FIBRIN

functions in a similar manner as the intrinsic system previously described. Thus, factor Xa is considered the pivotal substance, activated by both intrinsic and extrinsic systems (Biggs, 1972).

The sequence of reactions is illustrated in Figure 2.

Stage Two - Conversion of Prothrombin to Thrombin

The thromboplastin generated from stage one acts on prothrombin, converting it to a highly proteolytic enzyme - thrombin.

Stage Three - Conversion of Fibrinogen to Fibrin

Thrombin acts on fibrinogen, converting it to fibrin by splitting off four small fragments - the fibrinopeptides (Blomback, 1967). The remaining monomers polymerize to form an insoluble fibrin network. Thrombin also activates factor XIII (fibrin stabilizing factor), which in the presence of calcium ions, catalyzes the formation of firm chemical bonds between the fibrin molecules, resulting in a strong stable clot (Lorand, 1965). The sequence of reactions are schematized in Figure 3.

Coagulation Balance In Vivo

The previously described schema of blood coagulation does not include the complex system of inhibitors (anticoagulants) which destroy molecules of activated factors. These substances are of great significance, but are poorly documented and little understood due to the complexity of their nature and function. Without these inhibitors the clotting mechanism could be activated adventitiously, causing the circulating blood to clot in vivo (Biggs, 1972). Thus, the normal coagulation system may be

FIGURE 2

Stage I of coagulation showing the sequence of events leading to the formation of thromboplastin.

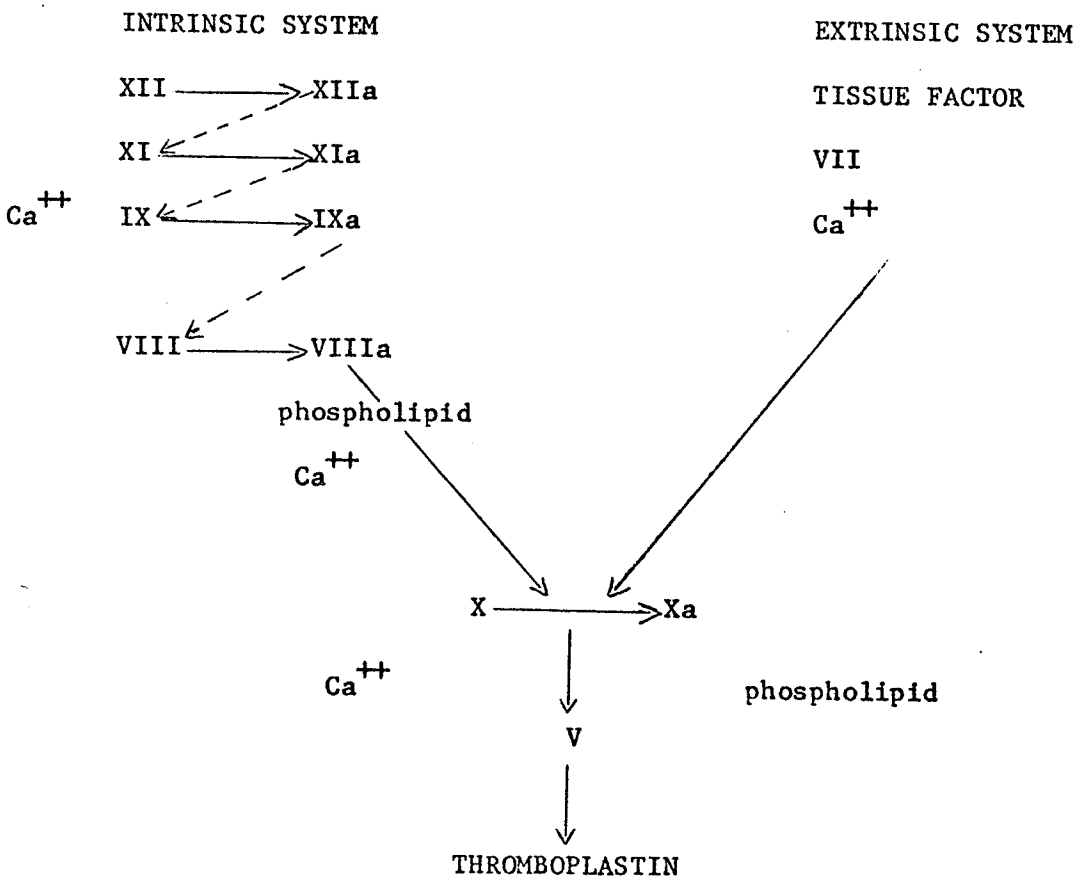
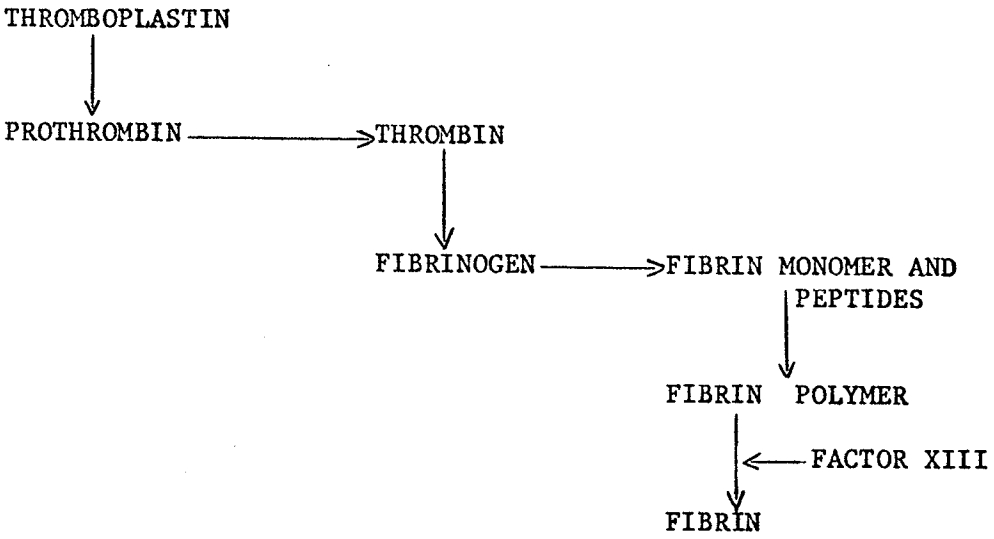

INTRINSIC SYSTEM
XII
XIIa
XI
XIa
Ca++
IX
IXa
VIII
VIIIa
phospholipid
Ca++
EXTRINSIC SYSTEM
TISSUE FACTOR
VII
Ca++
X
Xa
Ca++
phospholipid
V
THROMBOPLASTIN

FIGURE 3

The sequence of reactions in stages two and three of coagulation, resulting in the formation of fibrin.


THROMBOPLASTIN
PROTHROMBIN
THROMBIN
FIBRINOGEN
FIBRIN MONOMER AND PEPTIDES
FIBRIN POLYMER
FACTOR XIII
FIBRIN

considered to exist in a delicately balanced state of equilibrium in vivo. This equilibrium may be lost, resulting in hypercoagulability or hypocoagulability, depending on the nature of the disequiliberating forces. The dynamic nature of the coagulation system is illustrated in Figure 4.

The Fibrinolytic Mechanism

At this point it is appropriate to discuss the fibrinolytic system and the suggested pathways for fibrin dissolution.

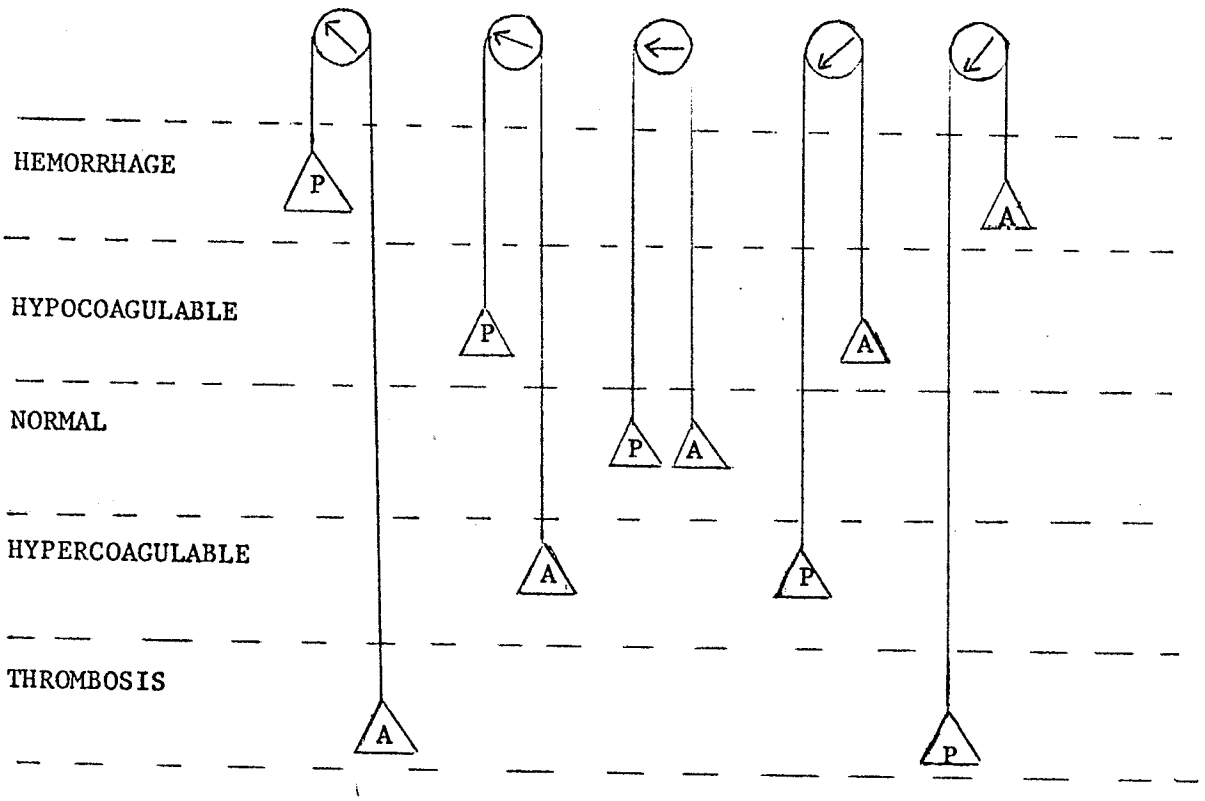
Astrup (1970) stated "clot formation is an unphysiologic endpoint". Thus, although fibrin formation constitutes a normal process of repair, its retention is considered pathological. The true physiologic endpoint is that of clot dissolution or lysis.

Activation of the fibrinolytic system involves the conversion of plasminogen into a highly proteolytic enzyme - plasmin. This conversion is an irreversible enzymatic reaction, involving the splitting of a number of peptide bonds and is brought about by plasminogen activator. The source of activator is relatively unclear, but is thought to arise in part from the vascular endothelium - a cholinergic effector mechanism possibly being involved (Kwaan, Lo and McFadzean, 1957). Activator may be either directly released from the tissue or may be converted from an inactive precursor-proactivator. The active enzyme-plasmin, exerts proteolytic activity on fibrin, fibrinogen, factor V (Johnson, Fletcher, McCarty and Tillett, 1957) and factor VIII (Ouchi, Simpson, Belco and Warren, 1961).

FIGURE 4

FIGURE 1

Concept of coagulation balance in vivo (Alexander, 1971)



A = Anticoagulants

P - Procoagulants

When plasminogen is slowly converted into plasmin, it is usually neutralized by antiplasmins (Norman, 1958). However, rapid activation of plasminogen may temporarily overwhelm the antiplasmin mechanism, resulting in the digestion of fibrin, fibrinogen and other coagulation substrates.

When fibrinogen or fibrin are digested by plasmin, breakdown products are formed. These products retain antigenic similarity to fibrinogen, and may be demonstrated in the serum of clotted blood by immunological techniques such as immunodiffusion (Ferreira, Murate, and Ferri, 1964) or tanned red cell hemagglutination inhibition immunoassay (Merskey, Kleiner and Johnson, 1966; Israels, Rayner, Israels and Zipursky, 1968).

Components of the fibrinolytic system are illustrated in Figure 5.

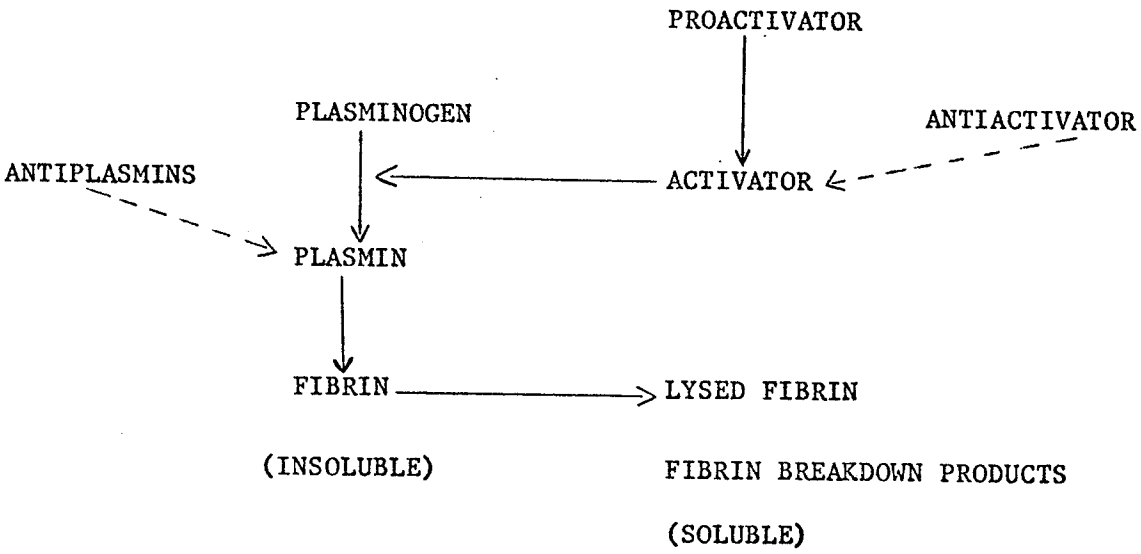
Inter-Relationships Between Coagulation and Fibrinolysis

Astrup (1958) hypothesized the existence of a dynamic equilibrium between thromboplastic and fibrinolytic activities as a means of maintaining vascular patency. Although teleologically attractive, this hypothesis cannot be empirically evaluated due to methodological limitations. It is acknowledged, however, that some degree of equilibrium must exist if thrombosis or hemorrhage is to be avoided.

Although the coagulation and fibrinolytic mechanisms depend on separate groups of plasma proteins, there is evidence which suggests that both systems can be activated by similar mechanisms (Iatridis and Ferguson, 1961). In addition, coagulation

FIGURE 5

The fibrinolytic mechanism. Solid arrows indicate activation. (Dotted arrows indicate inhibition).


PROACTIVATOR
PLASMINOGEN
ANTIACTIVATOR
ANTIPLASMINS
ACTIVATOR
PLASMIN
FIBRIN
LYSED FIBRIN
(INSOLUBLE)
FIBRIN BREAKDOWN PRODUCTS
(SOLUBLE)

factors V, VIII, II and I are susceptible to the proteolytic action of plasmin (Johnson et al, 1957; Ouchi et al., 1961).

Fibrin or fibrinogen breakdown products have been found to be potent inhibitors of coagulation (Fletcher, Alkjaersis, and Sherry, 1962). These products may inhibit the thrombin - fibrinogen - fibrin conversion (Kowalski, Budzynski, Kapec, Latallo, Lipinski and Wegrzynowicz, 1964), or may interfere with the polymerization of fibrin monomer (Alkjaersig, Fletcher and Sherry, 1962). Fibrin clots formed in the presence of breakdown products have been found to be structurally defective (Bang, Fletcher, Alkerjaersig and Sherry, 1962). In addition, fibrin or fibrinogen breakdown products have been shown to inhibit platelet aggregation (Jerushalmy and Zucker, 1966).

On the basis of this evidence, an interaction between the coagulation and fibrinolytic systems is apparent.

Intravascular Coagulation

Under some pathological conditions, the blood may become markedly hypercoagulable, with increases in platelet agglutination and/or various coagulation factors. Such states may eventually result in a condition known as disseminated intravascular coagulation, in which fibrin clots are formed and deposited within the vascular system.

Paradoxically, severe hemorrhage may result when the balance between coagulation and fibrinolysis is tipped in the direction of excessive coagulation. With continuation of clotting in vivo, the factor levels gradually become depleted, causing

decreased levels of fibrinogen, prothrombin, factors V, VIII and circulating platelets. This state is usually accompanied by fibrinolysis, and the breakdown products which are released may further interfere with coagulation in some of the ways previously described.

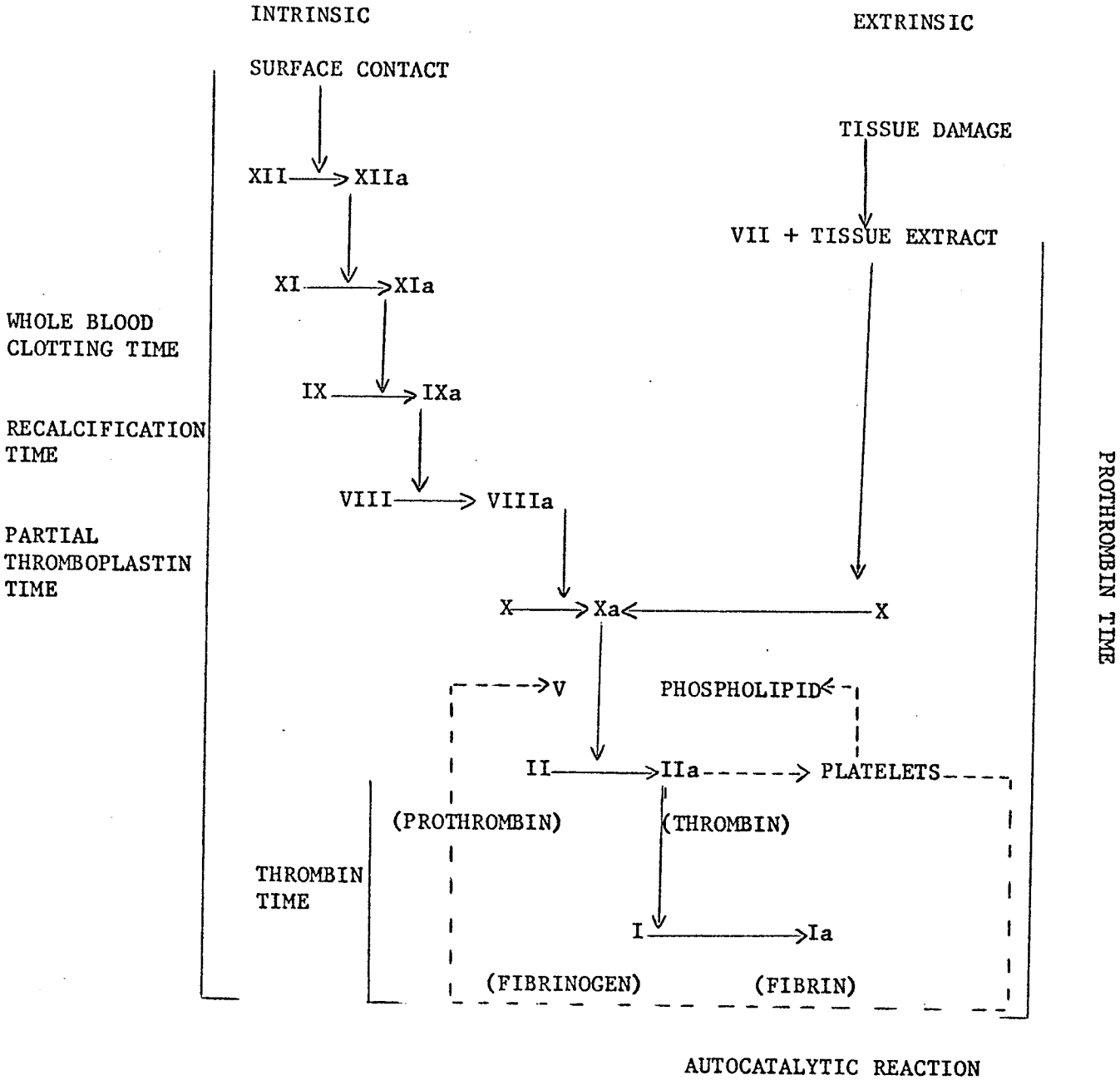
Tests of Coagulation Function

Since fibrinogen is the only coagulable protein, and thrombin the only substance capable of converting it to fibrin, all coagulation tests are necessarily based on the thrombin - fibrinogen - fibrin reaction (Biggs, 1969). Test systems have, however, been devised to measure specific components of the clotting mechanism, by using appropriate substrate mixtures. For example, phase one (formation of thromboplastin) may be bypassed by the addition of thromboplastin to the test system. Such a system would thus assess the activity of stages two and three (prothrombin time). Similarly, the final stage of the system may be selectively studied by bypassing the first two stages (thrombin time). These examples illustrate the logic underlying the majority of coagulation tests which are presented schematically in Figure 6.

The first attempt at a standardized coagulation assay was the technique of whole blood clotting time (WBCT) described by Lee & White (1913). This technique basically involves placing whole blood in glass or siliconized tubes and recording the time required for coagulation. This test thus reflects changes at any stage of the coagulation mechanism since all the stages must be passed through before a clot may be observed. Using the cascade hypothesis as a conceptual model, one may infer the relative

FIGURE 6

Enzyme cascade and tests of clotting function.
Modified from Denson and Biggs (1972).


INTRINSIC
EXTRINSIC
SURFACE CONTACT
TISSUE DAMAGE
XII → XIIa
VII + TISSUE EXTRACT
XI → XIa
WHOLE BLOOD CLOTTING TIME
IX → IXa
RECALCIFICATION TIME
VIII → VIIIa
PARTIAL THROMBOPLASTIN TIME
X → Xa ← X
V
PHOSPHOLIPID
II → IIa → PLATELETS
(PROTHROMBIN)
(THROMBIN)
THROMBIN TIME
I → Ia
(FIBRINOGEN)
(FIBRIN)
PROTHROMBIN TIME
AUTOCATALYTIC REACTION

contributions of the various factors to the overall system. Thus, the reactions involved in the initial stage will necessarily be the slowest and will make the greatest overall contribution to the time required for coagulation.

Despite its widespread use as an index of coagulability, this test is extremely insensitive. Results are influence by a number of technical variables such as the method of blood collection, the diameter of the glass tubes. The amount of handling or agitation can greatly decrease the clotting time due to increased enzyme-surface interactions (Tocantins and Kazal, 1964) thereby creating the impression of hypercoagulability. Denson and Biggs (1972) have stated "the major effects of the technical factors means that the test cannot be used for recording small differences" (p. 287). Furthermore, since most problems of technique serve to shorten the clotting time, interpretations of "hypercoagulability" based on results of the whole blood clotting time (WBCT) may be seriously questioned.

Relevance of the Problem

If environmental stressors induce alterations in the hemostatic and fibrinolytic mechanisms, such findings may have important implications.

Although the development of coronary disease has a multi-factorial etiology, psychosocial conditions have been shown to be important predisposing factors (Friedman & Rosenmann, 1959). Under acute emotional stress, and in emotionally tense, highly competitive driving individuals, the production of

catecholamines may be very high. These may produce a variety of effects which contribute to thrombogenesis.

Numerous anecdotal and scientific reports suggest the importance of stress in precipitating myocardial infarction (Dreyfuss, 1959; Jenkins, 1971; Keith, 1966; Rees & Lutkins, 1967; Weiss, Dlin, Rollin, Fisher & Bepler, 1957). Such reports are supported by recent studies (Haft & Fani, 1973 (a) (b) which demonstrate that stress-induced secretion of sympathetic catecholamines may cause intravascular platelet aggregates within the coronary tree.

In a series of experiments, Haft et al., (1973 (a)) found that rats subjected to the stress of cold showed deposition of platelet aggregates in the small vessel of the heart. Subsequent studies (Haft et al., 1973 (b)) in which stress was induced by electroshock or immersion in hot water reported essentially the same results. On the basis of these and other findings, the authors concluded that stress-induced platelet aggregates may occlude arteries which have been previously narrowed by atherosclerosis, resulting in the precipitation of acute myocardial infarction.

The influence of stress on the hemostatic mechanism thus represents an important area of study, and research within this area is worthy of further investigation.

Statement of the Purpose of the Study

The purpose of this study was thus to investigate the effects of stress on the blood coagulation system. Due to ethical

considerations, the experimental subjects were rats. However, the use of an animal analogue study provides stricter experimental control of possible extraneous variables.

In contrast to the crude insensitive method used in previous studies, reliable sensitive methods of measurement were employed. Thus it was possible to study the effects of stress on each stage of coagulation.

Stress was induced by restraint, cold, and restraint-cold in combination. Immobilization by restraint is considered to be a suitable routine procedure for the production of alarm reactions (Chubaty & Jaques, 1963; Selye, 1936), and if care is taken to avoid physical injury to the animal, is believed to induce primarily emotional stress. This procedure causes strong autonomic activity (urination and defecation) with resultant changes in a variety of physiological functions (Brodie & Hanson, 1963; Selye, 1946).

The effects of restraint stress may be potentiated by the addition of other stressors such as cold (Senay & Levine, 1967). It is thus hypothesized that the dual stress of restraint-cold will induce greater changes in blood coagulation than the separate effects of restraint or cold.

Since alterations in coagulation platelet activity and fibrinolysis have been induced by dietary means (Beard and Hampton, 1966; Davidson, Howard & Gresham, 1961; Renaud & Lecompte, 1970), all animals in the present study were maintained on normal diets of laboratory rat chow.

CHAPTER 2

The study was conducted as a series of five experiments. Each successive experiment attempted to expand the previous observations and to test their reliability. Experiment one was a pilot study. Experiments two and three sought to demonstrate the reliability of a stress-induced alteration in blood coagulability, whereas experiments four and five attempted to demonstrate how these changes occurred over time.

Experiment 1

Subjects

Eighteen mature male albino rats of the Sprague-Dawley strain, ranging in weight from 450-555 gms were used. Animals were randomly assigned to one of three groups, each containing six subjects, on the basis of body weight. All animals were kept under standard laboratory conditions and were maintained on diets of Purina laboratory rat chow (Ralston Co.) and water ad libitum, until the time of the experiment.

Apparatus

Plastic adjustable restraint holders (Fisher Scientific #1-280-10) were used to produce immobilization stress. Restraint volume was adjusted to the size of the animal by placing the door panel in one of three available positions. The panel was held in place by means of tape and/or rubber bands. In this device animals were unable to turn around, but had limited movement. (Figure 7).

Cold-stress was induced by placing the animals of the appropriate experimental group in a walk-in refrigerator at 26°F.

Method

Three groups of animals were designated as follows:

Group I control group, Group 2 restraint-cold stress, Group 3 restraint stress. Animals from the restraint group (Group 3) were subjected to immobilization stress for a period of six hours. Those of the restraint-cold group (Group 2) were subjected to the dual stress of immobilization-cold for a period of three hours. Control animals received no stress treatment and remained in their home cages until the time of blood collection.

The experiment was conducted over a period of three days. Animals were subjected to their respective treatments in triplets, two from each group being examined simultaneously. Blood samples were drawn at approximately the same time each day (late afternoon) in an attempt to minimize physiological fluctuations due to circadian rhythms.

Collection of blood sample by cardiac puncture

At the end of the designated stress period each animal was gently placed in an ether jar and lightly anaesthetized. The approximate position of the heart was located by palpation of the heartbeat, then the apex of the heart further identified as the central focus of strongest pulsations.

A clean sterile needle (20 gauge, 1 inch) attached to a 10 ml. sterile plastic syringe(B-D Luer lock, catalogue #801L/S) was cleanly inserted into the heart, and approximately 8 ml. of blood was quickly withdrawn, Figure 8. An aliquot of the blood (4.5 ml.) was immediately placed in a plastic tube containing one-tenth volume of acid-citrate anticoagulant, carefully inverted three to four times to assure proper anticoagulant mixing, then placed in a beaker of melting ice in the refrigerator. The remaining 3 ml. of blood was placed in a tube containing 0.1 ml. 2 Molar epsilon-

aminocaproic acid (EACA) and 50 units thrombin. This sample was then allowed to clot for 2 hours at 37°C.

Strict attention was paid to details of the above-mentioned procedure, since a clean puncture with minimal contamination with tissue juices is prerequisite for the reliability and validity of the results. If the initial puncture was unsuccessful, the needle was completely withdrawn from the animal's chest cavity. A new needle and syringe were then employed for the second attempt.

Since blood coagulation in the rat occurs relatively quickly, speed was an essential component of the blood collection procedure. The maximum allowable time for the collection of the blood sample was one minute. Cases in which the blood was slow in coming were terminated after one minute and the then available volume of blood was processed as previously described, using proportionately reduced volumes of anti-coagulant. If the total volume of blood was considered to be insufficient for further testing (less than 4 ml.), a second puncture was immediately attempted using a new needle and syringe. These time limitations were considered to be crucial for optimal results. Failure to adhere to such limitations would result in coagulation of the blood in vitro, with the inevitable loss of the plasma sample for further assay.

Processing of blood samples

Samples of anticoagulated blood (the plasma sample) were placed in beakers of melting ice which were then packed in a styrofoam cooler filled with ice cubes. Maintenance of the blood at such low temperatures is a necessary step which serves to prevent the loss of labile factors (such as Factors VIII and V) thereby

FIGURE 7

Rat in restraint cage.

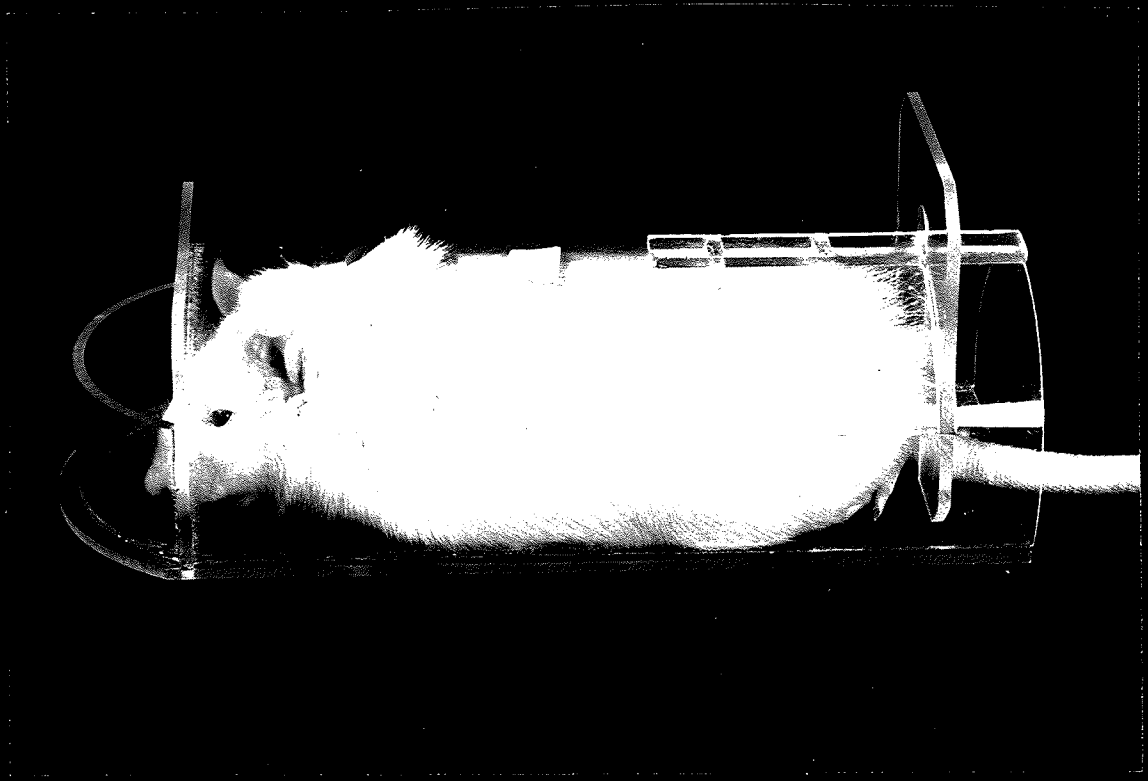
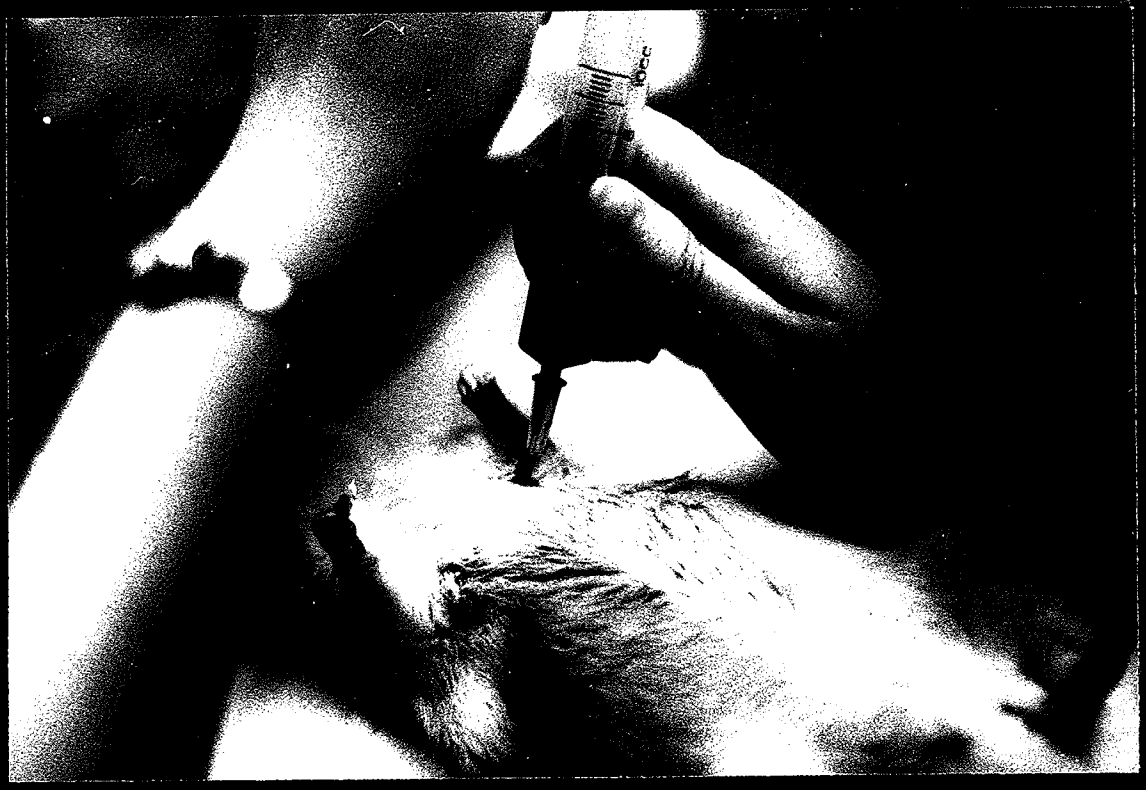


FIGURE 8

Withdrawal of blood by cardiac puncture.



ensuring the validity of the results. Serum samples were maintained at 37°C during transportation.

Immediately upon arrival at the Coagulation laboratory, the plasma samples were centrifuged at 12,000 r.p.m. for 15 minutes, using a Model B-20 International Refrigerated centrifuge. The plasma was then drawn off into small plastic tubes using a siliconized Pasteur pipette, labelled, and stored in a deep freeze at -35°C until required for later testing. Each sample was subdivided into five aliquots, thereby ensuring that the samples would not be subjected to repeated freezing and thawing in subsequent assays.

The samplesof serum were incubated at 37°C for two hours, then centrifuged at room temperature at 3,000 r.p.m. for 5 minutes. The supernatant serum was drawn into glass tubes, appropriately labelled, then stored at -35°C for later testing.

Figure 9 summarizes schematically the major steps in the processing of the sample.

Assay procedures

All coagulation tests were performed in duplicate under standardized conditions, using new glass tubes 12 x 17 mm. (catalogue #60825-405, Johns Co.).

Prothrombin times were performed on plasma by a modification of Quick's (1935) procedure with commercially prepared thromboplastin, using the first appearance of fibrin threads as the end-point. (Illustrated in Figure 10). Partial thromboplastin times (P.T.T.) were performed by a modification of the Proctor & Rapaport (1961) procedure, using celite for

FIGURE 9

Schematic summary of the major steps in processing the blood sample.

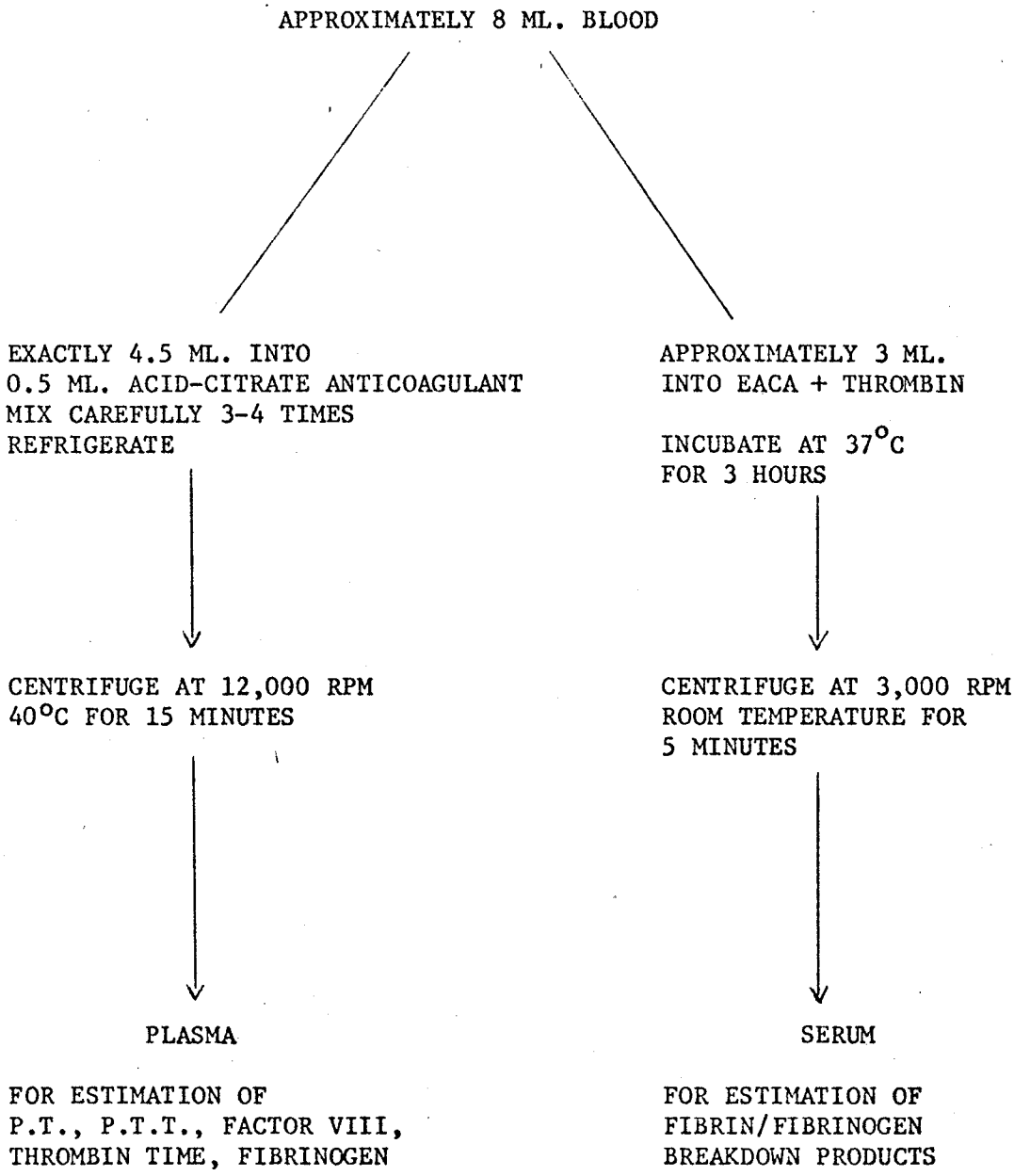

APPROXIMATELY 8 ML. BLOOD
EXACTLY 4.5 ML. INTO
0.5 ML. ACID-CITRATE ANTICOAGULANT
MIX CAREFULLY 3-4 TIMES
REFRIGERATE
CENTRIFUGE AT 12,000 RPM
40°C FOR 15 MINUTES
PLASMA
FOR ESTIMATION OF
P.T., P.T.T., FACTOR VIII,
THROMBIN TIME, FIBRINOGEN
APPROXIMATELY 3 ML.
INTO EACA + THROMBIN
INCUBATE AT 37°C
FOR 3 HOURS
CENTRIFUGE AT 3,000 RPM
ROOM TEMPERATURE FOR
5 MINUTES
SERUM
FOR ESTIMATION OF
FIBRIN/FIBRINOGEN
BREAKDOWN PRODUCTS

FIGURE 10

Fibrin end-point of coagulation tests

maximal activation of contact factors. Thrombin times were determined at 1 unit/ml. thrombin, using a modification of the Fletcher et al. (1959) method in which the plasma is added to a titration mixture containing calcium and the clotting time determined following the addition of thrombin. Fibrinogen levels were measured by the Astrup, Brakman & Nissen (1965) procedure, and Factor VIII assayed by the method of Niemetz & Nossel (1969). Details of these procedures are presented in Appendix I.

Each assay was performed within the same testing session, using the same stock of reagents. This was necessary in order to minimize variability in test results due to minor fluctuations in the activity of test reagents and/or temperature of the water bath. Thus, for example, all PTTs were conducted on each sample of the control and experimental groups on the same day, using the same reagents. This allowed for meaningful comparisons of test results between groups.

Factor VIII assays could not be performed on all samples on the same experimental day, due to the length of the procedure. In this case six samples, two from each group,were assayed each day over successive days. The dilution curves for the percentage concentration of Factor VIII activity were obtained from frozen samples of pooled normal human plasma representing 100% activity.

Statistical analysis

The data of the three groups were analyzed by a one way analysis of variance. Multiple comparisons among means were tested by the Scheffe (1953) procedure.

Results

Mean values for each of the main dependent measures for each of the three groups are summarized in Table 1-1. Analysis of variance of each of the measures revealed statistically significant differences in Prothrombin time ($F = 14.62$; df : 2,13, $p<.001$);

Table 1-1 reveals that average prothrombin time of the group subjected to the dual stress of restraint-cold was significantly longer than the control group ($F = 11.59$, df = 2,13, $p<.005$).

Partial thromboplastin times of the restraint-cold group were significantly prolonged, in comparison to controls ($F = 8.2$, df : 2,13, $p<.005$). This prolongation was partially due to the concomitant reduction in level of Factor VIII between restraint-cold and control groups ($F = 3.65$, df : 2,13, $p<.10$). Although a slight prolongation of the P.T.T. was also evident in the restraint group in comparison to controls, this difference was not statistically significant. However, a significant reduction in Factor VIII level was found between restraint vs control groups ($F = 4.23$, df : 2,13, $p<.05$).

These findings demonstrated that the dual stress of restraint-cold induced a state of hypocoagulability. This was evident from the prolongation of the P.T.T. and lowered levels of Factor VIII. A similar trend was apparent in the restraint group, although the changes were of a lesser magnitude.

In contrast to the stress-induced prolongation of P.T., P.T.T., and reduction of Factor VIII, the thrombin times of the restraint-cold group were significantly shorter in comparison to

controls ($F = 3.74$, $df : 2,13$, $p<.10$).

The Ouchterlony double-diffusion technique for the presence of fibrin/fibrinogen breakdown products was negative for all samples of control and experimental groups.

TABLE 1-1

MEAN AND STANDARD DEVIATIONS OF P.T.,P.T.T., FACTOR VIII, THROMBIN TIME AND FIBRINOGEN FOR CONTROL AND EXPERIMENTAL GROUPS.

	CONTROL		RESTRAINT-COLD		RESTRAINT	
	MEANS	S.D.	MEANS	S.D.	MEANS	S.D.
P.T.	12.75	.61	14.7	.45	13.7	.67
P.T.T.	21.67	.98	32.7	7.33	24.8	3.4
FACTOR VIII	154.33	91.64	51.14	29.19	43.1	39.93
THROMBIN TIME	44.42	5.97	36.8	2.17	43.1	4.42
FIBRINOGEN LEVEL	419.05	247.5	315.24	39.43	323.46	23.61

TABLE 1-2

F RATIO OF SCHEFFE MULTIPLE COMPARISONS
BETWEEN CONTROL AND EXPERIMENTAL GROUPS

	CONTROL VS RESTRAINT-COLD	CONTROL VS RESTRAINT
P.T.	F = 11.59 ($p<.005$)	F = 3.58 ($p<.10$)
P.T.T.	F = 8.2 ($p<.005$)	F = 0.65 (NS)
FACTOR VIII	F = 3.65 ($p<.10$)	F = 4.23 ($p<.005$)
THROMBIN TIME	F = 3.74 ($p<.10$)	F = 0.11 (NS)

Experiment 2

Subjects

Twenty four male albino rats of the Wistar strain, ranging in weight from 260-330 gms were used. Animals were randomly assigned to one of four groups of six subjects each, on the basis of weight. All animals were fed standard diets of Purina rat chow and water ad libitum until the time of the experiment.

Method

Four groups of animals were employed - control group (Group I), restraint-cold group (Group 2), restraint group (Group 3), cold group (Group 4). The duration of stress for Groups 2,3 and 4 was three hours. Throughout the course of the experiment, animals were subjected to their respective treatments in sets of four, one set being studied at the same point in time. Collection and processing of blood samples and assay procedures were identical to those described in Experiment 1. Data were analyzed by analysis of variance (2 x 2 factorial).

Results

Table 2-1 summarizes the mean values for each of the measures for control and experimental groups. Prothrombin times showed a significant interaction of restraint and cold ($F = 8.24$, df : 1,19, $p<.001$). However, differences between groups were not sufficiently great to be of practical significance, since the values were close to the range of reproducibility of the technique.

Partial thromboplastin times were significantly prolonged by restraint ($F = 9.47$, df : 1,19, $p<.01$), cold ($F = 4.75$, df : 1,19, $p<.05$) and the interaction of restraint-cold ($F = 9.47$, df : 1,19, $p<.01$).

In contrast to findings of the previous experiment, Factor VIII levels showed no differences between groups. This may have resulted from either age or strain differences, or from the increased difficulty in performing the cardiac punctures, since these animals were at least 200 gms lighter than those of the previous experiment. The difficulty in obtaining the blood samples may have explained the presence of small amounts of cryoprecipitate in some of the plasma samples. Although cryoprecipitate (a precipitate which forms at low temperatures) is observed in a variety of conditions, including intravascular coagulation, the possibility remains that it may have resulted from minimal contamination of the sample with tissue juices. In such case, the Factor VIII levels may have been erroneously high.

Although thrombin times showed a slight reduction due to the interaction of restraint-cold, these differences were not significant. Summary tables of the analysis of variance for P.T.,P.T.T. are presented in Tables 2-2,2-3.

In summary, the pattern of results of the restraint-cold group showed a similar trend to those found in Experiment 1, although the differences were of considerably lesser magnitude. The interaction of restraint-cold was found to induce a relatively hypocoagulable state, evidenced by a prolongation in the P.T. and P.T.T.

TABLE 2-1

MEANS AND STANDARD DEVIATIONS FOR P.T., P.T.T., FACTOR VIII, THROMBIN TIME AND FIBRINOGEN FOR CONTROL AND EXPERIMENTAL GROUPS

	CONTROL		RESTRAINT-COLD		RESTRAINT		COLD	
	MEANS	S.D.	MEANS	S.D.	MEANS	S.D.	MEANS	S.D.
P.T.	12.92	.74	14.42	.58	12.08	.20	13.8	.76
P.T.T.	19.42	2.06	25.67	3.57	19.67	1.94	18.6	3.54
FACTOR VIII	104.67	31.4	96.79	74.2	141.5	79.29	148.0	78.87
THROMBIN TIME	33.0	1.67	29.17	1.16	35.6	4.9	33.8	2.17
FIBRINOGEN LEVEL	264.8	23.86	302.8	121.8	279.85	42.08	293.14	55.5

TABLE 2-2

ANALYSIS OF VARIANCE : P.T. DATA

SOURCE	df	MS	F
Restraint	1	0.673	0.18
Cold	1	14.85	40.73**
Restraint x Cold	1	3.005	8.24*
Within cells	19	0.365	

* $p<.01$

** $p<.001$

TABLE 2-3

ANALYSIS OF VARIANCE : P.T.T. DATA

SOURCE	df	MS	F
Restraint	1	76.81	9.47*
Cold	1	38.55	4.75**
Restraint x Cold	1	66.38	8.19**
Within cells	19	8.109	

* $p<.05$

** $p<.01$

Experiment 3

Subjects

Twenty four mature male albino rats of the Sprague-Dawley strain ranged in weight from 480-560 gms. Animals were randomly assigned to one of four groups on the basis of weight. All animals were maintained on normal diets and were given food and water ad libitum until the time of the experiment.

Method

Four groups of animals were used. Designation of the groups was the same as that of Experiment 2. Animals of the stress groups were exposed to their respective treatments for a period of three hours.

Collection and processing of blood samples was as previously described. However, an additional 1 ml. of blood was drawn for enumeration of platelets. Blood for this test was placed in a tube containing disodium ethylenediamine tetracetate (E.D.T.A.) and mixed carefully. Platelets were counted by the direct method of Brecher & Cronkite (1950) using a hemocytometer and phase-contrast microscopy (Zernike, 1942). Details of the method are described in Appendix 1.

Results

The mean values of each of the measures for control and experimental groups are presented in Table 3-1.

Platelet counts were slightly reduced by the interaction of restraint-cold, although these differences were not significant.

Partial thromboplastin times showed a significant

prolongation due to restraint ($F = 10.63$, df : 1,19, $p<.005$), cold ($F = 12.08$, df : 1,19, $p<.005$) and the interaction of restraint-cold ($F = 11.7$, df : 1,19, $p<.005$). Concomitant reductions in Factor VIII were found, with significant main effects for restraint ($F = 11.71$, df : 1,19, $p<.005$), cold ($F = 6.61$, df : 1,19, $p<.05$) and the restraint x cold interaction ($F = 6.76$, df : 1,19, $p<.05$). Summary tables of the analysis of variance of P.T.T. and Factor VIII are presented in Tables 3-2 and 3-3.

Thrombin times showed a similar trend to that of the previous experiments. Significant reductions in thrombin time were found for restraint ($F = 3.29$, df : 1,19, $p<.10$), cold ($F = 3.23$, df : 1,19, $p<.10$) and the restraint x cold interaction ($F = 3.35$, df : 1,19, $p<.10$).

Subsequent tests were performed in an attempt to provide an explanation for the reduced thrombin times. Immunoelectrophoretic analysis of plasma samples of restraint-cold and control groups showed no differences in electrophoretic mobility patterns. The protamine-sulfate gelation test (Gurewich & Hutchinson, 1971) for the presence of soluble fibrin complexes were negative for all samples of the restraint-cold and control groups. Measurement of anti-thrombin III levels by immunodiffusion failed to demonstrate quantitative differences between stressed vs control animals.

The main findings of this experiment, namely the prolongation in P.T.T. reduced levels of Factor VIII, and shortened thrombin times were not only consistent with those of the previous experiments but, in addition provided convincing evidence of a stress-induced hypo-coagulable state.

TABLE 3-1

MEANS AND STANDARD DEVIATIONS FOR THE MAIN DEPENDENT MEASURES FOR CONTROL AND EXPERIMENTAL GROUPS

	CONTROL		RESTRAINT-COLD		RESTRAINT		COLD	
	MEANS	S.D.	MEANS	S.D.	MEANS	S.D.	MEANS	S.D.
P.T.	13.58	.38	14.92	1.59	14.5	.71	13.75	.42
P.T.T.	20.08	1.24	43.67	16.02	19.5	.87	20.25	2.89
FACTOR VIII	103.67	40.79	18.03	10.32	92.0	28.85	104.17	44.9
THROMBIN TIME	43.58	5.11	34.00	1.76	42.4	2.04	41.58	2.71
FIBRINOGEN LEVEL	371.6	58.95	405.25	73.6	371.78	80.43	360.33	58.63
PLATELET COUNT (10^3)	98.5	14.24	90.67	18.49	108.25	11.78	115.83	15.2

TABLE 3-2

ANALYSIS OF VARIANCE : P.T.T. DATA

SOURCE	df	MS	F
Restraint	1	748.04	10.63*
Cold	1	849.55	12.08*
Restraint x Cold	1	822.85	11.07*
Within cells		70.34	

* $p<.005$

TABLE 3-3

ANALYSIS OF VARIANCE : FACTOR VIII DATA

SOURCE	df	MS	F
Restraint	1	13723.63	11.71**
Cold	1	774.2	6.61*
Restraint x Cold	1	7921.67	6.76*
Within cells	19	1171.74	

* $p<.05$

** $p<.01$

Experiment 4

Subjects

Twenty four mature male albino rats of the Sprague-Dawley strain ranged in weight from 420-580 gms. Animals were randomly assigned to one of four groups on the basis of weight. Food and water were available ad libitum until immediately prior to the experiment.

Method

In an attempt to determine whether the hypocoagulability observed in the previous experiments resulted from an earlier state of hypercoagulability which had perhaps culminated in intravascular coagulation, animals of the experimental groups were subjected to varying durations of restraint-cold stress.

The following four groups were used. Group 1 - control group, Group 2 - restraint-cold stress for 10 minutes duration, Group 3 - restraint-cold stress for 30 minutes duration, Group 4 - restraint-cold stress for 4 hours duration,

Collection, processing and assaying of blood samples was as previously described.

Results

Mean values and standard deviations for each of the groups are presented in Table 4-1.

Analysis of variance revealed statistically significant differences between groups in Prothrombin time ($F = 4.75$, df : 3,19, $p<.05$), partial thromboplastin time ($F = 10.29$, df : 3,19, $p<.05$), Factor VIII level ($F = 36.64$, df : 3,19, $p<.001$) and Thrombin time ($F = 24.25$, df : 3,19, $p<.001$).

Table 4-2 shows that increasing durations of stress resulted in progressive changes in the coagulation parameters. No trend towards hypercoagulability was evident over the periods tested. The P.T.T. showed a gradual prolongation over time, with greatest differences occurring at 4 hours ($F = 22.13$, df : 3,19, $p<.001$), Figure 11. Factor VIII levels showed a concomitant reduction ($F = 26.45$, df : 3,19, $p<.001$). However, Figure 12 reveals that precipitous reductions in Factor VIII occurred after only 10 minutes stress ($F = 21.34$, df : 3,19, $p<.001$).

Thrombin times showed a progressive shortening over time - a trend which was consistent with previous experiments. This shortening became most pronounced in the 4 hour stress group, with an average overall reduction of 15 seconds in comparison to controls ($F = 24.75$, df : 3,19, $p<.001$). This trend is presented in Figure 13.

Animals subjected to stress for 4 hours had slightly lower platelet counts than control animals. However, such differences were not significant.

As in previous experiments, no fibrin/fibrinogen breakdown products were detectable in the stress or control groups.

TABLE 4-1

MEANS AND STANDARD DEVIATIONS FOR P.T., P.T.T., FACTOR VIII, THROMBIN TIME, FIBRINOGEN LEVELS AND PLATELET COUNTS FOR CONTROL AND EXPERIMENTAL GROUPS

	CONTROL GROUP MEANS	S.D.	STRESS 10 MINS MEANS	S.D.	STRESS 30 MINS MEANS	S.D.	STRESS 4 HRS MEANS	S.D.
P.T.	14.75	.27	15.0	.32	14.6	.65	15.58	.58
P.T.T.	21.83	2.11	25.0	3.69	26.9	1.54	36.92	8.08
FACTOR VIII	187.5	44.7	49.75	26.42	35.4	21.65	27.67	18.1
THROMBIN TIME	46.17	3.63	36.92	3.4	39.2	2.56	31.42	1.86
FIBRINOGEN LEVEL	367.35	74.8	359.42	53.53	328.72	34.22	343.02	38.1
PLATELET COUNT (10^3)	112.0	25.0	107.17	18.84	104.0	24.14	97.0	11.08

TABLE 4-2

F RATIOS OF SCHEFFE MULTIPLE COMPARISONS
BETWEEN CONTROL AND EXPERIMENTAL GROUPS

	CONTROL VS GROUP 2	CONTROL VS GROUP 3	CONTROL VS GROUP 4	GROUP 2 VS3	GROUP 2 VS4	GROUP 3 VS4
P.T.	F=.277(NS	F=.09(NS)	F=3.06(p<.05)	F=.65(NS)	F=1.49(p<.25)	F=3.873(p<.05)
P.T.T.	F=.98 (NS)	F=2.27(p<.25)	F=22.13(p<.001)	F=.32(NS)	F=13.8(p<.001)	F=8.87(p<.001)
FACTOR VIII	F=21.34(p<.001)	F=23.66(p<.001)	F=26.45(p<.001)	F=.21(NS)	F=.33(NS)	F=.61(NS)
THROMBIN TIME	F=9.73(p<.001)	F=5.02(p<.01)	F=24.75(p<.001)	F=.54(NS)	F=3.44(p<.05)	F=6.26(p<.005)

FIGURE 11

Progressive prolongation in the P.T.T. with increasing durations of restraint-cold stress.

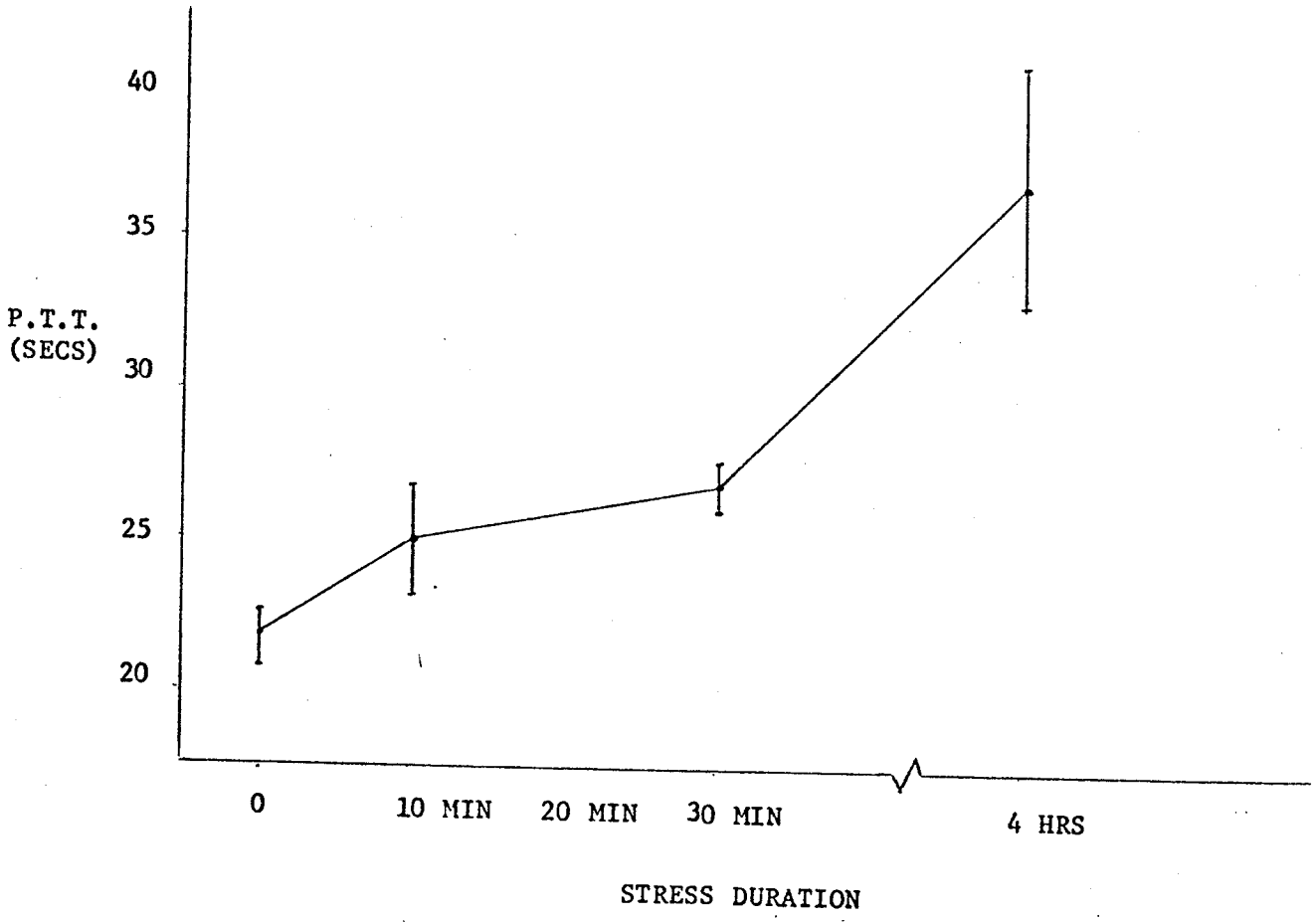

40
35
P.T.T.
(SECS)
30
25
20
0
10 MIN
20 MIN
30 MIN
4 HRS
STRESS DURATION

FIGURE 12

Reduction in Factor VIII level with increasing durations of restraint-cold stress.

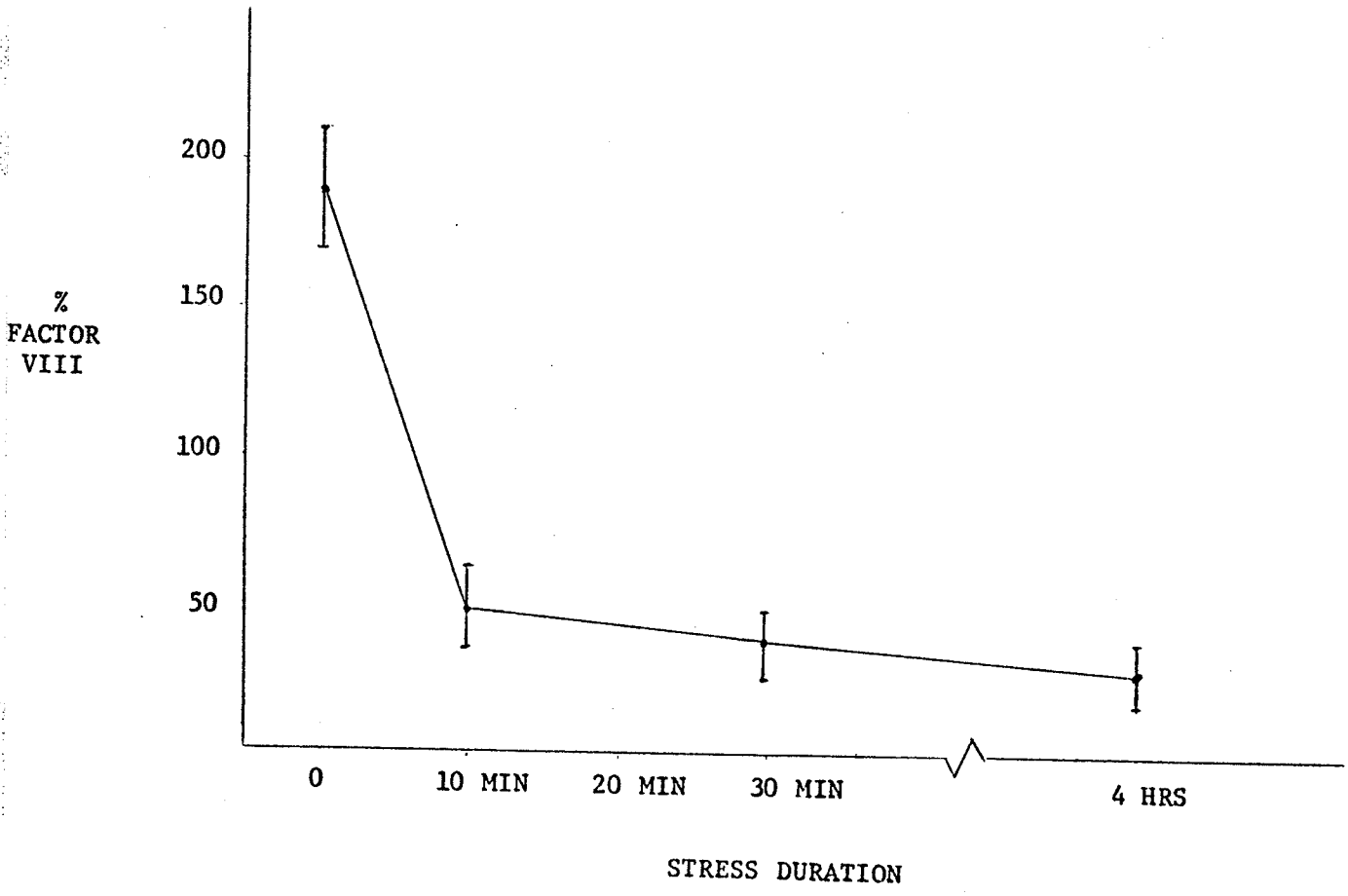
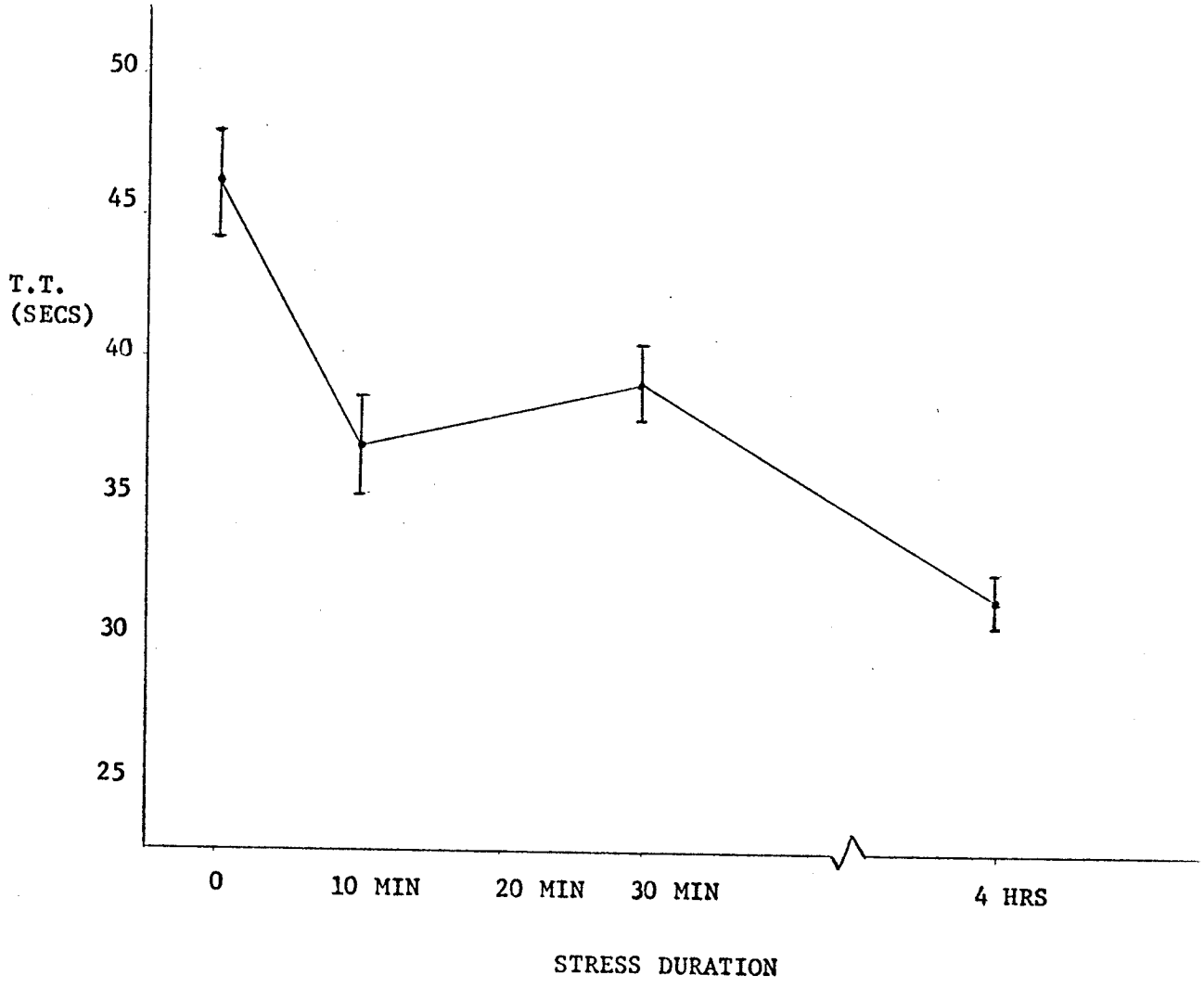

200
150
100
50
% FACTOR VIII
0
10 MIN
20 MIN
30 MIN
4 HRS
STRESS DURATION

FIGURE 13

Reduction in thrombin time with increasing durations of restraint-cold stress.


50
45
T.T.
(SECS)
40
35
30
25
0
10 MIN
20 MIN
30 MIN
4 HRS
STRESS DURATION

Experiment 5

Subjects

Twelve mature male albino rats of the Sprague-Dawley strain ranging in weight from 540-580 gms. were used. Animals were randomly assigned to one of two experimental groups. Food and water were available ad libitum until immediately prior to the experiment.

Method

The experiment was conducted to determine whether prolonged periods of immobilization stress would produce changes in coagulability similar to those induced by short periods of restraint-cold stress. Animals of both experimental groups were thus subjected to prolonged restraint for either 18 hours or 24 hours duration.

Since throughout the course of all previous experiments, minimal variability in coagulation parameters was found among unstressed animals, subjects of the control group from Experiment 4 also served as normal controls for the present experiment. However, in order to allow for meaningful comparisons between groups, the samples from the control group were retested at the time the samples of the experimental groups were being assayed. This controlled for minimal fluctuations due to potency of test reagents.

Results

Table 5-1 summarizes the mean values for each dependent measure for control and experimental groups. Statistically

significant differences between groups were found in Prothrombin time ($F = 13.32$, df : 2,14, $p<.01$), partial thromboplastin time ($F = 7.31$, df : 2,14, $p<.01$), Factor VIII level ($F = 17.93$, df : 2,14, $p<.001$), thrombin time ($F = 17.87$, df : 2,14, $p<.001$) and fibrinogen level ($F = 5.61$, df : 2,14, $p<.025$).

As in previous experiments, stress resulted in a hypocoagulable state, evidenced by a prolongation in the P.T.T. and concomitant reductions in Factor VIII. Table 5-2 indicates that this trend was most apparent following 18 hours restraint. Partial thromboplastin times of this group were significantly longer than those of controls ($F = 6.94$, df : 2,14, $p<.01$). Factor VIII levels of the 18 hour and 24 hour stress groups were significantly reduced in comparison to the control group ($F = 15.9$, df : 2,14, $p<.001$; $F = 10.22$, df : 2,14, $p<.001$ respectively).

Thrombin times of both experimental groups were significantly shorter than controls, with average reduction around 10 seconds. Such reductions, in addition to being of statistical significance are of considerable practical significance. In contrast to the four previous experiments, animals subjected to restraint showed an increase in fibrinogen levels. This increase was attributed to the prolonged duration of the stress treatment.

In this experiment animals subjected to 18 hours restraint showed relatively greater differences in coagulation indices in comparison to animals subjected to restraint for 24 hours. Such findings may reflect some tendency towards adaptation and the organisms attempts to restore hemostatic equilibrium.

TABLE 5-1

MEANS AND STANDARD DEVIATIONS FOR P.T., P.T.T., FACTOR VIII, THROMBIN TIME, FIBRINOGEN LEVEL AND PLATELET COUNTS FOR CONTROL AND EXPERIMENTAL GROUPS

	CONTROL GROUP		RESTRAINT 18 HRS		RESTRAINT 24 HRS	
	MEANS	S.D.	MEANS	S.D.	MEANS	S.D.
P.T.	14.75	.27	13.6	.42	14.58	.49
P.T.T.	21.83	2.11	29.2	5.06	25.58	2.22
FACTOR VIII	187.5	44.47	39.0	16.72	74.0	55.62
THROMBIN TIME	45.25	3.8	36.3	1.35	36.75	2.54
FIBRINOGEN LEVEL	367.37	74.8	534.06	87.54	484.37	94.05
PLATELET COUNT (10^3)	112.0	25.0	94.2	22.45	106.0	9.59

TABLE 5-2

F RATIOS OF SCHEFFE MULTIPLE COMPARISONS
BETWEEN CONTROL AND EXPERIMENTAL GROUPS

	CONTROL VS 18 HRS RESTRAINT	CONTROL VS 24 HRS RESTRAINT	18 VS 24 HRS RESTRAINT
P.T.	F = 11.06 (p<.005)	F = .27 (NS)	F = 8.03(p<.005)
P.T.T.	F = 6.94(p<.01)	F = 1.06 (NS)	F = 2.73(p<.10)
FACTOR VIII	F = 15.9(p<.001)	F = 10.22(p<.005)	F = .88 (NS)
THROMBIN TIME	F = 13.65(p<.001)	F = 13.54(p<.001)	F = .03 (NS)
FIBRINOGEN LEVEL	F = 5.16(p<.025)	F = 2.79(p<.10)	F = .46 (NS)

CHAPTER 3

DISCUSSION

The main findings of the study may be summarized as follows:

(1) In all five experiments, stress induced changes in blood coagulability. Animals subjected to the stress of restraint-cold showed significant prolongations in Prothrombin times, Partial thromboplastin times, and concomitant reductions in levels of Factor VIII. The single exception occurred in Experiment 2, in which Factor VIII levels remained normal. However, as previously discussed, these findings may have resulted from a variety of factors such as age differences, strain differences, or differences in body weight.

(2) In contrast to the stress-induced prolongation in the P.T.T. and reduced levels of Factor VIII, the Thrombin times of animals subjected to the stress of restraint-cold and prolonged restraint were considerably shorter than controls. Although in Experiments 1,2 and 3, the significance levels were not impressively high, the average reductions observed in Experiments 4 and 5 were highly significant.

(3) Increasing durations of restraint-cold stress caused progressive alterations in coagulation parameters. Changes in P.T.T., Factor VIII, and Thrombin time were evident after only 10 minutes of restraint-cold, with greatest changes occurring at 4 hours.

(4) Platelet counts of some experimental groups were slightly reduced in comparison to controls, although the differences were not significant.

(5) Tests for the presence of fibrin/fibrinogen breakdown products were negative for all animals of control and experimental groups

in all five experiments.

The coagulation findings will now be discussed since subsequent discussions are primarily dependent on their interpretation.

Interpretation of coagulation findings

On superficial examination the stress-induced hypocoagulability observed in the present study appears to be inconsistent with the stress-hypercoagulability hypothesis of previous investigators (Cannon & Grey, 1914; DeLong et al., 1959; Dreyfuss, 1956; Friedman & Uhley, 1959; Macht, 1952; Uhley & Friedman, 1959). However, closer examination of the results suggests that the observed hypocoagulability was preceded by an earlier state of hypercoagulability which culminated in intravascular coagulation. As previously described, once the coagulation process is triggered, it initiates a series of reactions which lead to the generation of thrombin and the deposition of fibrin within the vascular system. Intravascular activation of the coagulation mechanism during disseminated intravascular coagulation (DIC) results in gradual depletion of platelets and plasma factors, especially of Factor VIII (Penick, Roberts, Webster & Brinkhous, 1958), activation of the fibrinolytic system, and the appearance of fibrin breakdown products (McKay, 1965). However, episodes of DIC may vary in severity. Low grade DIC may proceed at a much slower rate and some chronic forms of DIC may be difficult to detect, since consumption of clotting factors and platelets is fully compensated by their increased synthesis.

The prolonged P.T.T. and reduced levels of Factor VIII observed in the present study are consistent with previous reports

of coagulation changes in DIC (McKay, 1965; Penick et al., 1958). However, the present findings are not typical of this phenomenon in all respects - for example, fibrin breakdown products were not detectible in the sera of stressed animals. Previous authors (Prose, Lee & Balk, 1965) have established that the phagocytic activity of the reticuloendothelial system (RES) constitutes an important mechanism in clearing small fibrin aggregates which circulate in the blood during states of diffuse low grade DIC. Thus, in the present study, fibrin breakdown products may have been undetected due to clearing from the circulation by the RES.

An alternative explanation for the absence of fibrin breakdown products is based on an intrinsic methodological difficulty. Immunological techniques used for the detection of FDP are based on the removal of fibrinogen from the sample prior to assay. Serum is used,as plasma fibrinogen will react with the antiserum. However, during clotting the large breakdown products of fibrinogen which are also coagulable (Fragment X, Fletcher & Alkjaersig, 1973) as well as fibrinogen are removed. Thus, in the present study FDP may not have been detectible either because of clearance by the RES, or because of their incorporation within the blood clot.

In severe DIC the thrombin time is typically prolonged, primarily because of depletion of fibrinogen to low levels and/or to the anti-thrombin activity of some FDP (Alkjaersig et al., 1962; Fletcher et al., 1962). However, in the present study the thrombin times of stressed animals were shorter than those of controls. Since the thrombin time measures the final phase of coagulation,

namely the conversion of fibrinogen to fibrin by the action of thrombin, the test is thus sensitive to a limited number of variables. These include the absolute concentration of the substrate fibrinogen, the rate of the fibrinogen-fibrin conversion, or levels of anti-thrombin 3 - a natural inhibitor of thrombin.

In the present study fibrinogen levels of stressed animals were similar to those of controls. Thus the shortened thrombin times could not be attributed to increased levels of fibrinogen. Recent investigators (Banerjee, Sahni, Kunan & Arya, 1974) have found significant correlations between thrombin times and levels of anti-thrombin 3, and have attributed shortening of the thrombin time to reduced levels of anti-thrombin 3. However, in this study subsequent measurement of anti-thrombin 3 levels by immunodiffusion failed to demonstrate quantitative differences between stressed vs control animals.

In a further attempt to explain the shortened thrombin times, attention was drawn to the possible presence of soluble fibrin complexes (SFC). These high molecular weight complexes have been found to have clot promoting properties, which include shortening of the thrombin time (Arnesen, 1973; Bang & Chang, 1974) and have been considered indicative of hypercoagulability or incipient thrombosis (Bang, Hansen, Smith, Latallo, Chang & Mattler, 1973; Hansen, Bang, Barton & Mattler, 1972). When thrombin acts on fibrinogen, fibrinopeptides are released, and the remaining portion of the molecule is referred to as fibrin monomer. These monomers polymerize and form a clot. In vivo, with a slow formation of fibrin monomer, complexes are formed with

native fibrinogen. These complexes of fibrin monomer with fibrinogen are soluble and can be removed from the circulation by the RES without compromising the patency of the small blood vessels. If these complexes are present in an in vitro plasma specimen, because part of the fibrinogen has already been acted upon by thrombin, the time required for formation of a visible clot in the thrombin time test will be reduced in comparison to normal.

A number of test systems for the measurement of soluble fibrin complexes have recently been devised. One of such tests - the protamine-sulfate gelation test (Gurewich & Hutchinson, 1971) employed in an attempt to demonstrate the presence of SFC in the plasma of stressed animals yielded negative results. However, some authors (Bang & Chang, 1974) have seriously questioned the specificity and sensitivity of this test, and have cautioned about problems of false negative results. It was thus concluded that the shortened thrombin times observed in stressed animals suggested the presence of soluble fibrin complexes and indicated ongoing intravascular coagulation.

In summary, the prolonged P.T.T.'s, reduced levels of Factor VIII and shortened thrombin times of the stressed animals were interpreted as indices of an earlier state of hypercoagulability which had culminated in episodes of low grade intravascular coagulation. Results of Experiment 4 (which tested the effects of various durations of restraint-cold stress) showed reduced Factor VIII levels after only 10 minutes of stress. This implies that if hypercoagulability did in fact occur, it must have occurred within a period of

10 minutes of stress.

Mechanisms of Stress effects on Coagulation

The mode of action of stress on blood coagulation remains speculative. However, it is postulated that stress in general and restraint in particular induced alterations in coagulation through activation of the pituitary-adrenal axis.

Numerous studies (Bliss, Midgeon, Branch & Samuels, 1956; Mason, 1959; Selye, 1946; Smelik, 1960) have established the importance of the pituitary-adrenal system in the stress response, and augmented levels of catecholamines in response to diverse stressors have been reported (Elmadgian, Hope & Lamson, 1957; Friedman, St. George, Byers & Rosenman, 1960; VonEuler, 1960; Young & Gray, 1965). Rosenman & Friedman (1957) noted that individuals exhibiting a behaviour pattern associated with increased vulnerability to coronary heart disease showed increased excretion of catecholamines in conjunction with accelerated coagulation during periods of stress.

Other evidence is based on the findings of investigators who have studied the effects of adrenaline on the coagulation system. Ingram & Vaughan-Jones (1966) demonstrated that adrenaline induced accelerated blood coagulation, by increasing the activity of Factor VIII. This effect was abolished by prior administration of beta-adrenergic blocking agents. Adrenaline has also been reported to cause in vivo increases in platelet number (McClure, Ingram & Jones, 1965) and platelet aggregation (O'Brien, 1963). Such findings suggest that stress-induced changes in coagulation may be mediated through the release of adrenaline.

Further support is provided by studies employing stimulation of diencephalic and hypothalamic neural areas (Gunn & Hampton, 1967; Kubantseva, 1973). Hypercoagulability resulting from stimulation of the posterior hypothalamus was attributed to a sympathetic-like response from stimulation of the predominantly adrenergic structures within this region (Kubantseva, 1973).

Acute or sustained sympathetic adrenergic overactivity elicited by emotional stressors may thus cause increased release of circulating catecholamines. This may result in intravascular platelet aggregation with release of platelet constituents. When platelets aggregate they accelerate the coagulation process, primarily because platelet phospholipid which is released is essential for later phases of the coagulation reaction (the interaction between Factors IX and VIII and between Factors V and X). In addition, a number of coagulation factors, namely fibrinogen, Factors V, XIII, XII and XIII are adsorbed on the platelet surface. Thus platelet aggregates may initiate or accelerate the coagulation process in vivo.

These events may also be potentiated by stress-induced elevations in plasma lipids (Gottschalk, Cleghorn, Gleser & Iacano, 1965). Studies suggest that long chain free fatty acids can exert a thrombogenic effect when lipid levels rise rapidly in response to humoral stimuli (Connor, Hook & Warner, 1969). Such evidence may provide a further link between stress and the tendency to thrombosis.

Psychological influence on Blood Coagulation

The findings of the present study in conjunction with

clinical observations demonstrate that emotional stress may affect the coagulation system. Thus this study extends present-day conceptions of psychosomatic interrelationships.

Stressful life situations which produce anxiety and fear have been consistently associated with hypercoagulability (Dreyfuss, 1956; Macht, 1952; Kast & Zweibel, 1954; Schneider, 1956; Vuori, 1950). Such situations have included the stress of awaiting electroshock therapy (Kast & Zweibel, 1954; Vuori, 1950) blood donation (Macht, 1952) and final examinations (Dreyfuss, 1956; Schneider, 1951). Other attempts to characterize stress in terms of the individual's perspective or symbolic representation of the situation rather than as a function of externally imposed conditions have identified feelings of conflict, anxiety and apprehension in relation to accelerated coagulation. Groups differentiated on the basis of an anxiety-worry-fear complex showed differential sensitivity to the occurrence of emotional stress (Friedman & Rosenman, 1959). This behaviour pattern was subsequently defined as characterized by intense sustained drive to achieve, extreme competitiveness, feelings of urgency and extreme emotional arousal. This constellation of factors was considered to be largely responsible for the hypercoagulability and thrombotic tendency under conditions of stress.

Psychotic or neurotic states appear to be unrelated to changes in coagulability. However, transient mental states of the individual have caused various deviations from the normal coagulation parameters (Aleksandrowicz et al., 1964). Thus, transient states of fear or anxiety may cause relative hyper-

coagulability regardless of nosologic diagnosis, whereas psychomotor depression appears to be associated with relative hypocoagulability (Aleksandrowicz et al., 1964; Schneider, 1951).

Experimental animal analogue studies provide similar types of evidence. Fear induced by unavoidable shock has been associated with blood hypercoagulability (DeLong, 1959; Friedman et al., 1959; Uhley et al., 1959). Haft and Fani (1973a,b) observed acute agitation and extreme arousal in stressed animals in whom intravascular platelet aggregates were found.

In the present study animals subjected to restraint, and restraint-cold stress showed evidence of extreme autonomic arousal, such as urination and defecation during the stress treatment. Those of Experiment 3, which showed the greatest coagulation changes also manifested behavioural signs of extreme emotionality. Such animals reacted strongly to handling and to the restraint procedure and would often jump into the air to avoid being placed in the restraint device.

The findings of the present study support previous reports of stress-induced alterations in blood coagulability. Since coagulability is a factor in the pathogenesis of thrombosis the implications of these findings are important.

SUMMARY

The present study was undertaken in an attempt to resolve existing discrepancies regarding the influence of stress on blood coagulability. Refined sensitive methods of measurement were employed which permitted the assessment of stress effects on specific components of the blood coagulation mechanism.

In a series of five separate experiments, the effects of various stressors on the coagulation system of mature male rats were investigated. Results demonstrated that:

(1) Animals subjected to restraint-cold showed evidence of a severe hypocoagulable state characterized by prolongations in Partial Thromboplastin times and reduced Factor VIII activity.

(2) In contrast to the stress-induced prolongation in the P.T.T. and reduced levels of Factor VIII, the Thrombin times of stressed animals were considerably shorter than those of controls. Although the significance levels were not impressively high in Experiments 1,2 and 3, the average reductions observed in Experiments 4 and 5 were highly significant.

(3) Increasing durations of restraint-cold stress caused progressive alterations in coagulation parameters. Changes in P.T.T., Factor VIII and Thrombin time were evident after only 10 minutes of restraint-cold, with greatest changes occurring over the intervals tested.

(4) Platelet counts of some experimental groups were slightly reduced in comparison to controls, although differences were not statistically significant.

(5) Tests for the presence of fibrin/fibrinogen

breakdown products were negative for all subjects of experimental and control groups.

On superficial examination these results appeared to be inconsistent with the hypothesis that stress causes hypercoagulability. However, on closer examination the results suggested that the observed hypocoagulability was preceded by a state of hypercoagulability which culminated in intravascular coagulation.

Possible mechanisms of stress effects on blood coagulation were considered. It was postulated that stress-induced changes in coagulation may be mediated through the pituitary-adrenal axis and increased excretion of catecholamines.

Examination of the psychological states during which stress-induced changes in coagulation have resulted suggested that emotional states which are dominated by feelings of anxiety, fear, or hostility may contribute to the formation of thromboembolism.

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APPENDIX 1

EXPERIMENTAL METHODS

Acid-Citrate Anticoagulant for the Preparation of Plasma

The anticoagulant was made by mixing 2 parts of 0.1 Molar citric acid with 3 parts of 0.1 Molar sodium citrate. One volume of this solution was added to 9 volumes of blood (i.e. 0.5 ml. acid citrate was added to 4.5 ml. blood).

This anticoagulant is the optimal choice for processing samples of frozen plasma since it prevents the pH from rising on storage.

EACA and Thrombin for Preparation of Serum for Fibrin Breakdown Products

50 λ thrombin (1,000 units/ml.)

0.1 ml. 1 Molar EACA

3 - 5 ml. blood was added to the above mixture; resulting in immediate coagulation of the blood. The tube was incubated at 37°C for 3 hours, and the serum removed following centrifugation.

Procedure for Enumeration of Platelets (Brecher-Cronkite, 1950)

Blood was drawn in a red cell pipet to the 0.5 mark with great accuracy, then diluted to 101 with 1% ammonium oxalate. The diluted blood was then thoroughly mixed in a pipet shaker for 5 minutes.

The first four drops from the pipet were then expelled and discarded and the hemocytometer filled and covered with a Petri dish containing wet filter paper for 15 minutes. Counts were performed in duplicate using phase contrast microscopy and

high power magnification. Platelets, identifiable by their size and characteristic highly refractile silvery appearance, were counted within the whole of the finely ruled central area of the counting chamber.

Prothrombin Time
(Modification of Quick, 1935)

In this test tissue thromboplastin is added to citrated plasma, thus reactions of Stage 1 of coagulation are completely bypassed.

Procedure

0.2 ml. commercially prepared thromboplastin was incubated at 37°C in a glass tube for 3 minutes.

0.1 ml. prewarmed plasma sample was then blown into the bottom of the tube, a stopwatch simultaneously started, and a wire hook rapidly passed through the mixture. The presence of fibrin threads indicated the end-point.

Reproducibility of this test is ± 1.0 second.

Partial Thromboplastin Time
(Modification of Proctor & Rapaport, 1961)

In this test plasma is recalcified in the presence of a lipid reagent (cephalin) which supplies optimal platelet-factor like activity. Variability of contact activation is eliminated by the addition of celite for a standardized period of time.

Reagents

Cephalin diluted 1/100 with veronal buffer

Celite 0.7% in physiological saline

Calcium chloride 0.025 Molar.

Procedure

The partial thromboplastin celite reagent was prepared by mixing equal aliquots of diluted stock celite solution and diluted cephalin.

0.2 ml. of this partial thromboplastin-celite reagent was pipeted into a glass test tube. (Since the celite sediments rapidly, the reagent must be thoroughly mixed immediately before pipeting).

0.2 ml. test plasma was added, and the mixture incubated at 37°C for exactly 3 minutes 0.2 ml. prewarmed calcium chloride was then added and a stopwatch simultaneously started. A wire loop was then passed through the mixture until the end-point (the presence of fibrin) is detected.

This test is highly reproducible, and is considered to be the best available method for evaluating the overall integrity of the coagulation mechanism.

Thrombin Time
(Fletcher et al., 1959)

This test measures the ability of thrombin to convert fibrinogen to fibrin, thus the first two stages of coagulation are completely bypassed.

Reagents

Titration mixture (Seegers & Smith, 1942)

Thrombin (Parke-Davis) diluted with 0.85% sodium chloride to a concentration of 1 unit/ml. (Since this reagent is very unstable at room temperature it is prepared as a final step and is kept on ice during the procedure).

Procedure

0.3 ml. titration mixture.

0.1 ml. plasma sample.

0.1 ml. diluted thrombin. On addition of thrombin a stopwatch was started and a wire hook passed through the mixture. The presence of the first fibrin thread indicated the end-point.

The test is sensitive to levels of fibrinogen and to interference of the fibrinogen fibrin reaction by circulating anticoagulants of fibrinogen breakdown products.

Factor VIII Assay
(Niemetz & Nossel, 1969)

Reagents

2% celite in 0.85% sodium chloride.

cephalin diluted 1/100 in veronal buffer

citrate-saline (1 part 3.8% sodium citrate: 5 parts NaCl)

veronal buffer-citrate saline mixture (59:1)

Calcium chloride 7/20

Factor VIII deficient plasma.

Procedure

Standardized pooled normal human plasma was diluted 1/20, 1/50, 1/100 in citrate-saline-veronal buffer immediately prior to testing.

To an incubation tube was added the following:

0.1 ml. factor VIII deficient substrate

0.1 ml. plasma dilution

0.1 ml. 1/100 cephalin

0.1 ml. celite.

The contents of the tube were gently mixed, and incubated exactly 5 minutes. 0.1 ml. calcium chloride was then added, and a stopwatch simultaneously started. The tube was tilted for observation of the clot end-point.

Values for the pooled normal human plasma were plotted graphically on double log paper. These values represented 100% Factor VIII activity.

Samples of rat plasma were diluted and tested in exactly the same manner. Percentage Factor VIII activity was obtained by interpolation of the 100% reference curve.

Estimation of Fibrinogen (Astrup et al., 1965)

Reagents

Saline L-Ee : 50 mg L-lysine ethyl ester dihydrochloride in 100 ml. 0.15 Molar sodium chloride

2.5N sodium hydroxide

1N hydrochloric acid

L-tyrosine (Fisher) : 200 mg. in 1000 ml. 0.05N HCl

0.025 M. calcium chloride

20% W/v sodium carbonate

Phenol reagent (Harleco) diluted 1/10 with distilled water (prepared immediately prior to using).

Procedure

0.5 ml. plasma sample was added to 1 ml. saline L-Ea in a 12 x 17 mm. test tube, and clotted with 0.5 ml. 0.025 Molar $CaCl_2$ and 10 λ thrombin.

The solution was stirred with a glass rod (5 mm. diameter) roughened on one end. The rod was then slowly turned and the fibrin carefully wound around it. After one hour winding was resumed, and the fluid carefully squeezed from the fibrin, by pressing the clot gently against the wall of the tube. The clot was washed twice in saline L-Ee for 10 minutes, then was placed in a graduated conical centrifuge tube containing 1.0 ml. 2.5N NaOH. The fibrin was dissolved in the NaOH by placing the tube in a boiling water bath for 10 minutes.

The tube was then cooled and the mixture neutralized by addition of 2 ml. 1N HCl. The glass rod then was removed while being rinsed with distilled water to a total volume of 10 ml. An aliquot of 2.5 ml. was transferred to a second tube containing 1.5 ml. 20% Na_2CO_3. To this was added 2.5 ml. diluted phenol reagent. The mixture was left for 30 minutes at room temperature for full color development and absorbency read at 710 mμ on a Beckman D.B. Spectrophotometer, 1 cm. glass cell against a blank which was prepared without the clot.

Readings were converted to tyrosine values by interpolation on a standard reference curve.

APPENDIX 2

APPENDIX 2

EXPERIMENTAL DATA

EXPERIMENT 1

		PT	PTT	VIII	THROMBIN TIME	FIBRINOGEN
	1	13.0	21.0	290	43.5	288.6
	2	13.0	22.5	165	48.5	272.5
CONTROL	3	12.0	21.0	217	39.5	758.2
	4	12.0	22.0	61	35.5	203.8
	5	13.0	23.0	51	50.0	278.0
	6	13.5	20.5	142	49.5	713.3
	1	15.0	35.0	88	40.0	253.7
	2	15.0	41.0	32	37.0	353.3
RESTRAINT	3	14.0	21.0	14.7	36.0	331.3
COLD	4	14.5	32	51	37.0	300.5
	5	-	-	-	-	-
	6	15.0	34.5	70	34.0	336.9
	1	-	-	-	-	-
	2	14.0	21.5	113.5	43.5	356.6
RESTRAINT	3	13.0	25.5	17.0	43.0	314.5
	4	14.0	28.0	35.0	45.0	322.9
	5	14.0	28.0	22.0	48.0	331.3
	6	13.0	21.0	28.0	36.0	292.0

Values shown are averages of duplicate estimations

EXPERIMENT 2

		PT	PTT	VIII	THROMBIN TIME	FIBRINOGEN
	1	13.5	20.5	100	35.0	302.2
	2	14.0	23.0	155	35.0	263.9
CONTROL	3	12.0	18.0	93	31.0	247.1
	4	12.5	17.5	60	32.0	280.8
	5	12.5	20.0	120	32.5	235.9
	6	13.0	18.0	100	32.0	258.3
	1	14.5	21.5	122	28.5	247.1
	2	14.5	27.0	18	28.5	547.5
RESTRAINT	3	15.0	29.0	130	28.0	219.0
COLD	4	13.5	24.5	108	30.5	252.7
	5	14.0	22.0	200	29.5	269.6
	6	15.0	30.0	2.7	28.0	280.0
	1	12.0	19.5	95	32.0	207.8
	2	12.0	20.0	85	36.0	275.2
RESTRAINT	3	12.0	18.0	64	31.5	292.0
ALONE	4	12.0	22.0	280	45.0	320.1
	5	12.5	17.0	165	35.5	263.9
	6	12.0	21.5	160	33.0	320.1
	1	-	-	-	-	-
	2	13.0	18.5	50	33.5	219.0
COLD	3	13.5		270	33.0	365.0
ALONE	4	14.0	20.5	140	34.0	269.6
	5	13.5	23.0	130	31.0	325.7
	6	15	17.5	150	37.0	286.4

EXPERIMENT 3

		PLATELET COUNT (10^3)	PT	PTT	VIII	THROMBIN TIME	FIBRINOGEN
	1	124	13.5	21.0	135	42.0	483.0
	2	92	13.5	20.0	75	44.0	376.3
CONTROL	3	105	13.5	19.5	170	39.5	359.4
	4	84	14.0	18.0	92	47.0	308.9
	5	95	13.0	21.5	62	37.5	353.8
	6	91	14.0	20.5	88	55.5	348.2
	1	101	18.0	75.0	7.5	37.0	325.7
	2	94	14.5	44.0	23.0	32.0	409.9
RESTRAINT	3	99	14.5	32.5	35.0	32.5	409.9
COLD	4	61	14.0	36.0	8.5	34.0	539.1
	5	78	15.0	41.5	14.2	34.0	393.1
	6	112	13.5	33.0	20.0	34.5	353.8
	1	100	15.0	20.0	110	41.5	303.3
	2	125	15.0	19.5	120	41.5	320.1
RESTRAINT	3	100	15.0	20.0	50	46.0	505.4
	4	108	13.5	20.0	75	41.0	348.2
	5	-	14.0	18.0	105	42.0	381.9
	6	-	-	-	-	-	-
	1	108	14.0	19.0	85	43.0	336.9
	2	140	14.0	23.5	43.0	42.5	460.5
COLD	3	130	14.5	18.0	120	45.0	370.6
	4	106	13.0	24.0	72	37.0	314.5
	5	106	13.5	20.0	150	40.5	381.9
	6	106	13.5	17.0	155	41.5	297.6

EXPERIMENT 4

		PLATELET COUNT (10^3)	PT	PTT	VIII	THROMBIN TIME	FIBRINOGEN
	1	100	14.5	21.0	200	41.0	393.1
	2	91	14.5	19.0	190	46.5	339.7
CONTROL	3	115	15.0	21.5	200	48.0	494.2
	4	116	14.5	23.5	100	49.5	351.0
	5	158	15.0	25.0	210	42.5	359.4
	6	92	15.0	21.0	225	49.5	266.7
	1	142	15.0	22.5	25	33.5	370.6
	2	86	14.5	27.0	40	36.5	370.7
STRESS	3	97	15.0	21.5	77	42.0	359.4
10 MINS	4	104	15.5	24.0	50	37.5	280.8
	5	107	15.0	31.5	21.5	33.0	443.7
	6	107	15.0	23.5	85	39.0	331.3
	1	82	15.0	30.0	20	38.0	297.7
	2	85	15.5	28.0	30	43.5	325.7
STRESS	3	136	14.5	27.5	19	39.5	362.2
30 MINS	4	94	14.0	22.5	36	37.0	293.0
	5	123	14.0	26.5	72	38.0	365.0
	6	-	-	-	-	-	-
	1	111	16.5	28.5	60	34.5	336.9
	2	102	16.0	47.0	15	31.5	401.5
STRESS	3	82	15.5	35.0	32	32.5	348.2
4 HOURS	4	95	15.0	32.5	30	29.5	325.7
	5	87	15.5	47.0	9	30.5	359.4
	6	105	15.0	31.5	20	30.0	286.4

EXPERIMENT 5

		PLATELET COUNT (10^3)	PT	PTT	VIII	THROMBIN TIME	FIBRINOGEN
	1	100	14.5	21.0	200	39.5	393.1
	2	91	14.5	19.0	190	48.0	339.8
CONTROL	3	115	15.0	21.5	200	47.0	494.2
	4	116	14.5	23.5	100	45.5	351.0
	5	158	15.0	25.0	210	42.0	359.4
	6	92	15.0	21.0	225	49.5	266.7
	1	90	14.0	37.0	28	36.5	401.5
	2	120	14.0	30.5	17	35.0	516.7
RESTRAINT	3	80	13.0	29.0	45	35.5	595.3
18 HOURS	4	114	13.5	24.5	60	38.5	527.9
	5	67	13.5	25.0	45	36.0	628.9
	6	-	-	-	-	-	-
	1	103	14.5	23.5	170	40.0	443.6
	2	100	14.5	29.0	110	38.5	516.7
RESTRAINT	3	101	14.5	23.5	50	38.5	662.7
24 HOURS	4	103	14.5	23.0	24	34.5	435.2
	5	123	15.5	24.5	55	35.0	421.2
	6	-	14.0	24.0	35	34.0	426.8