THE STIMULATION OF FIBRINOLYTIC ACTIVITY IN MAN

by

J. D. Cash, B.Sc., M.B., Ch.B., M.R.C.P.E.

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To my Parents

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Statement in terms of the Ph.D. Regulation 13 of the University of Edinburgh

Some of the studies described in this thesis have been the subject of published articles:-

1. Effect of Moderate Exercise on the Fibrinolytic System in Normal Young Men and Women

   Cash, J. D. (1966). Brit. med. J., 2, 502.

2. The Effect of Mental Stress on the Fibrinolytic Reactivity to Exercise

   Cash, J. D. and Allan, A. G. E. (1967). Brit. med. J., 1,

3. The Fibrinolytic Response to Moderate Exercise and Intravenous Adrenaline in the Same Subjects

   Cash, J. D. and Allan, A. G. E. (1967). Brit. J. Haemat., 13, 376.

4. A Multichannel System for the Automatic Recording of Clot Lysis

   Cash, J. D. and Leask, E. (1967). J. clin. Path., (In Press).

INTRODUCTION

The fibrinolytic process is concerned with the enzymatic dissolution of fibrin. The end-product is a single proteolytic enzyme, plasmin, which is responsible for the action of fibrinolysis. The proteolytic actions of plasmin are protean but in the presence of inhibitors in the blood it attacks only fibrin, to which it is adsorbed (Macfarlane and Biggs 1948, Celander and Guest 1957). Plasmin is derived from a stable inactive precursor named plasminogen which is present in the globulin fraction of plasma. Conversion of plasminogen to plasmin usually requires an activator or activating system.

Recent research in the field of fibrinolysis has been centred upon the therapeutic application of exogenous activators in thrombo-embolic disease (McNicol and Douglas 1964) and the relationship between excess fibrinolysis to severe haemorrhage (Sharp 1964) and diminished fibrinolysis in various pathological states (Fearnley 1965). Despite this increased activity our knowledge of the physiological roles of the fibrinolytic enzyme system remains fragmentary. The studies outlined in this thesis represent a further contribution to narrow this important gap.

CHAPTER 1

THE DEVELOPMENT OF THE MODERN CONCEPTS OF THE FIBRINOLYTIC ENZYME SYSTEM

Over 200 years ago Morgagni (1761), in his famous work "De Sedibus et Causis Morborum," reported that blood remained fluid in cases of sudden death. In 1794 John Hunter reported fluidity of blood in the cadaver and Denis (1838) and Zimmerman (1846) observed that the fibrin of human blood, obtained by wet cupping, sometimes dissolved in the absence of bacterial contamination. Green (1887) found that fibrin lysed in saline would not clot again on addition of thrombin. Dastre (1893) using dog fibrin as a substrate for studies on gastric enzymes observed that the substrate appeared to digest itself and he proposed the term fibrinolysis for this phenomenon. Nolf (1905, 1908) stressed that fibrinolysis must be due to a proteolytic enzyme and that this process was a basic mechanism of cellular nutrition.

Almost 30 years elapsed before any further major advance was made when Tillett and Garner (1933) reported that rapid lysis occurred in plasma clots incubated with extracts of beta-haemolytic streptococci. It was assumed that the extract contained a proteolytic enzyme specifically adapted for fibrinogen and fibrin, but Milstone (1941) observed no lysis with this extract on purified fibrinogen unless small amounts of a serum fraction

containing globulin were added. Christensen (1945) and Kaplan (1944), independently, concluded that the streptococcal factor was an activator of a precursor proactivator, present in the euglobulin fraction of human blood plasma or serum. Christensen and MacLeod (1945) proposed a new terminology for the factors involved: the active proteolytic enzyme was named plasmin, the precursor became plasminogen, the streptococcal activator streptokinase and the inhibitor antiplasmin.

Fig. 1 summarises briefly the basic structure of the fibrinolytic enzyme system, as envisaged at present.

More recent developments had been centred upon the isolation of plasminogen and plasmin, the activation of plasminogen to plasmin and a more detailed study of the inhibitor mechanisms, all of which will be discussed in the subsequent section.

A. THE COMPONENTS OF THE FIBRINOLYTIC ENZYME SYSTEM

1. PLASMINOGEN

Plasminogen is a β-globulin (Robbins and Summaria 1963) present in the euglobulin fraction of plasma (Milstone 1941). Major advances in isolation, purification and characterisation of human plasminogen were made with the acid extraction of Cohn Fraction III by Kline and

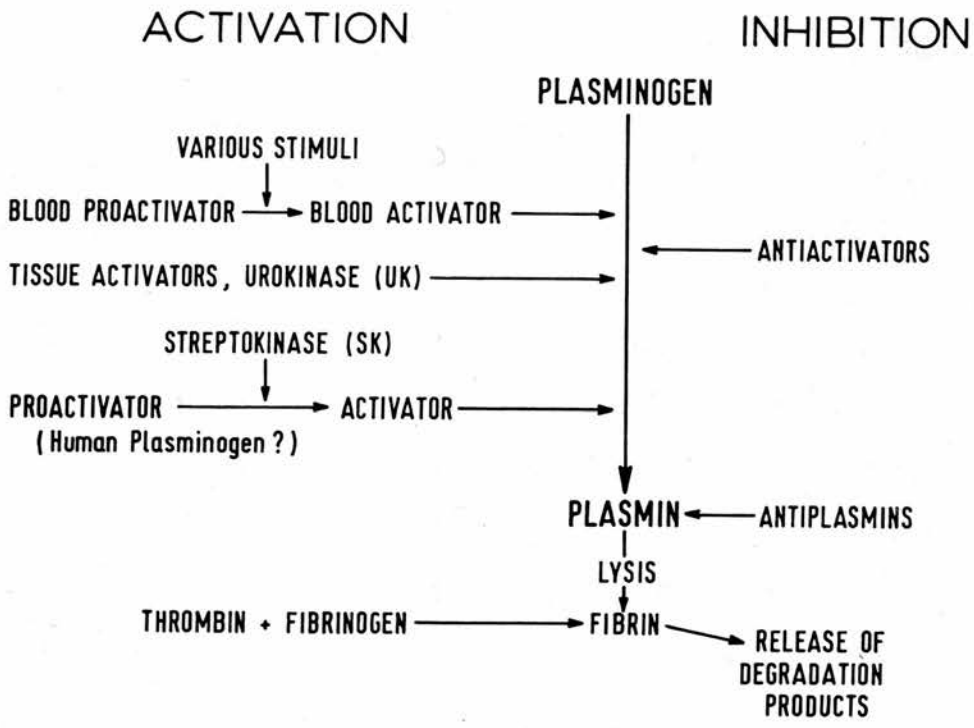

Fig.1. Simplified Scheme of Fibrinolysis.

his colleagues (Kline 1953, Kline and Fishman 1961). More recently, using chromatographic techniques, it has been possible to obtain preparations of higher specific activity (Alkjaersig 1960, Derechin et al. 1962, Robbins and Summaria 1964), but this has resulted in discordant observations on molecular weight (83,000 - 143,000), solubility characteristics, electrophoretic and ultracentrifugal behaviour, so that some preparations appeared to contain multiple species of plasminogen. In an effort to clarify this problem, Alkjaersig (1964) has concluded that the molecular characteristics of plasminogen may vary with the method of purification and that the molecule probably has a great deal of plasticity in terms of its secondary and tertiary structure.

The source of plasma plasminogen remains uncertain: Barnhart and Riddle (1963), on the basis of immunofluorescent studies, have suggested that the bone marrow eosinophil is a major source. It is also probable that the liver (Sherry 1965) and arterial intima (Todd 1964) may play significant roles.

Plasminogen is widely distributed throughout the body; its concentration in plasma has been estimated at 0.1 - 0.2 mg./ml. It has a tendency to co-precipitate with fibrin, so that significant quantities of plasminogen are incorporated into such deposits (vide infra).

2. PLASMIN

Despite the recent use of stabilizing agents, such as glycerol and lysine, in the activating mixtures of highly purified plasminogen preparations (Alkjaersig et al. 1958, Kline and Fishman 1964), the biophysical characteristics of plasmin await clarification, largely due to the problem of multimolecular species of plasminogen, discussed above. The biochemical activity, however, has been well characterised (Sherry et al. 1959b, Ablondi and Hagan 1960). It is a proteolytic enzyme of the endopeptidase type and can hydrolyse susceptible arginine and lysine bonds in proteins. Plasmin will digest fibrinogen in a manner and rate similar to its action on fibrin. It attacks a number of other plasma proteins including coagulation factors V and VIII. This dangerously protean proteolytic enzyme is kept in check by a large amount of circulating anti-plasmins (Norman 1966).

Plasmin breaks down fibrinogen and fibrin into small polypeptide moieties which may yet be shown to play a vital role in the proposed link between coagulation and fibrinolysis, for they can interfere with fibrin polymerisation (Alkjaersig et al. 1962) and platelet aggregation (Jerushalmy and Zucker 1966).

3. PLASMINOGEN ACTIVATORS

These are substances, either endogenous or exogenous,

which convert plasminogen to plasmin. They can be classified as physiological or non-physiological.

(a) Non-physiological activators

Streptokinase

This is an extracellular bacterial protein (molecular weight 50,000) derived from actively growing haemolytic streptococci - Lancefield's Group A, C and G. While it has no physiological role in man it has been used extensively both as a laboratory and therapeutic agent.

Streptokinase converts plasminogen to plasmin in human plasma but is unable to do so in bovine plasma, unless a trace amount of human serum or plasma is present. These findings led Geiger (1952) and Mullertz (1957) to conclude that streptokinase activation of plasminogen by streptokinase was an indirect one requiring the presence of a proactivator, normally present in human serum and plasma. Whether proactivator exists as a separate and distinct entity remains unsettled. However, data following the recent isolation of purified plasminogen (Kline and Fishman 1961, De Renzo et al. 1963, Alkjaersig 1964) favours the concept that streptokinase interacts with human plasminogen and/or plasmin to form a product with activator activity and that this reaction does not require the presence of an additional plasma factor. Until this hypothetical substance is isolated then its existence will

remain controversial.

Staphylokinase

Staphylokinase is present in cultured filtrates of some strains of staphylococci (Gerheim et al. 1948, Lack 1948). Staphylokinase activates plasminogen directly (Lewis and Ferguson 1951), but there appears to be considerable species difference in susceptibility (Cliffton and Cannamela 1952).

Non-specific plasminogen activation

There are a number of compounds, including peptone (Astrup and Olesen 1957), urea (Von Kaulla and Smith 1961), hydrophobic compounds (Von Kaulla 1961), heparin (Halse 1960) and protamine (Von Kaulla 1952), which have been shown to increase in vitro plasma fibrinolytic activity. Although it has generally been concluded that their primary mode of action is a destruction of anti-fibrinolytic substances, Olesen (1965) has postulated a cleavage of an activator-anti-activator complex by physico-chemical influences tending to interfere with protein interactions.

(b) Physiological activators

Tissue activators

Fischer (1946) first demonstrated that normal tissues

contained plasminogen activator, and this was subsequently confirmed by Astrup and Permin (1948), Tagnon and Palade (1950) and Lewis and Ferguson (1950): the latter workers considered it was located in the microsomal fraction. Lack and Ali (1964) have repeated this work and observed highest activity in the lysosomal fraction.

In an extensive study on the tissue content of plasminogen activator in different tissues, Albrechtsen (1957) found the uterus, adrenals, lymph nodes, prostate and lung tissues to contain high levels, but the normal liver failed to show activity.

Tissue activator was considered to exist in two forms, soluble and insoluble in saline, but it is probable that the soluble form may represent contaminating blood activator content. The insoluble fraction can be extracted by 2 M potassium thiocyanate. Activator prepared in this way is remarkably stable, resisting temperatures of 50° C. for 30 minutes over a wide pH range (Astrup and Sterndorff 1956). Further biochemical and biophysical characterisation has been impeded since the thiocyanate extraction procedure does not lend itself to further purification. Bachmann et al. (1964) have re-investigated this problem and, by using new chromatographic procedures, have achieved a major breakthrough in purification of plasminogen activator from pig heart. It is to be expected that in the next few years, when the new methods of purification have been fully exploited, a much greater degree of

information on the properties and mechanism of action will be available.

The biological significance of tissue activators remains to be elucidated. They may prove to make a significant contribution to the circulating activator (Maki et al. 1965) and also be essential in connective tissue repair mechanisms (Astrup 1966).

Urokinase

Williams (1951) confirmed the earlier observations of Macfarlane and Pilling (1947) that fibrinolytic activity was present in urine and further demonstrated it to be a plasminogen activator. It has been fairly extensively purified and characterised (Ploug and Kjeldgaard 1957, Sgouris et al. 1962, Bergstrom 1963), and appears to convert plasminogen to plasmin by first order kinetics (Kjeldgaard and Ploug 1957, Alkjaersig et al. 1958). Whether it represents cleared plasma activator and/or a renal product remains to be determined (Smyrniotis et al. 1959, Celander and Guest 1960, Holemans et al. 1966, Charlton 1966). The physiological function of urokinase is also speculative, but it may play a part in maintaining an unobstructed urine flow.

Although recent commercial products have been shown to be contaminated by thromboplastic materials (Douglas and McNicol 1964) it is currently undergoing extensive clinical trials as a thrombolytic agent.

Blood activators

An appreciation of the importance of handling withdrawn blood by low temperature techniques (Fearnley et al. 1952, Truelove 1953) and the development of more sensitive assays have resulted in observations which are generally interpreted as reasonable proof that trace amounts of plasminogen activator are normally present in the plasma of unstressed individuals (Fearnley and Lackner 1955, Sherry et al. 1959a, Flute 1960). Enhanced fibrinolytic activity, thought to be due to increased plasminogen activator, is associated with mental stress, exercise, adrenaline, ischaemia, shock, heat and pyrogens.

The source of plasma activator and the mechanisms associated with enhancement will be discussed in a subsequent section.

Activators of other body fluids

Plasminogen activators have been demonstrated in milk (Astrup and Sterndorff 1952), tears (Storm 1955), saliva (Albrechtsen and Thaysen 1955) and seminal fluid (Von Kaulla 1953). The role, if any, of activators in these fluids remains unexplored.

4. INHIBITORS

Fibrinolytic inhibitors can be classified into anti-activators and anti-plasmins.

Activator inhibitors

Plasma inhibitors

Naturally occurring activator inhibitors in plasma have been postulated (Lewis and Ferguson 1951, Jacobsson 1955, Mullertz 1957, Flute 1960, Paraskevas et al. 1962, McNicol et al. 1963) but, as yet, the assay methods do not preclude the possibility that they were not anti-plasmins.

Amino acid inhibitors

Following the earlier observation by Mullertz (1954) that the amino acids lysine and ornithine inhibit plasminogen activator, Okamoto (1954) demonstrated that the potency of ε -aminocaproic acid (E.A.C.A.) was considerably greater. E.A.C.A. is a competitive inhibitor of activator at concentrations of 10^{-4}M and a non-competitive inhibitor of plasmin at concentrations above 5×10^{-2}M (Alkjaersig et al. 1959a, Ablondi et al. 1959). E.A.C.A. has been used clinically for some years now but may be superseded by even more potent amino acids including | -(aminomethyl) cyclohexane -4- carboxylic acid (A.M.C.H.A.) and p-aminomethylbenzoic acid (P.A.M.B.A.). (Okamoto and Okamoto 1962, Lohmann et al. 1964.)

Plasmin inhibitors

Plasma anti-plasmins

Norman and Hill (1958) demonstrated an anti-plasmin in the α_2 globulin fraction reacting quickly as a competitive inhibitor and another slow reactor appearing in the α_1 fraction. More recent work by Moriau et al. (1964) has confirmed these findings and further suggested that a third anti-plasmin exists in the gamma fraction.

The physiological role of these inhibitors has hardly been explored, but it is assumed they play an important role in the control system which prevents excessive and dangerous fibrinolysis. On the other hand, in the presence of fibrin plasmin seems to favour inter-reaction with it rather than with the anti-plasmins (Norman 1966).

Platelet anti-plasmins

Although Johnson and Schneider (1953) demonstrated significant platelet anti-plasmin content this was not confirmed by Dudok de Wit (1964). Despite this discrepancy, platelet anti-plasmins may yet be shown to play an important role in the resistance to lysis of the in vivo thrombus.

Miscellaneous anti-plasmins

Several, apparently unrelated, substances have been shown to inhibit plasmin action: including basic amino

acids (Mullertz 1954), heparin (Von Kaulla and McDonald 1958), heavy metals (Kowalski et al. 1956), soya-bean trypsin inhibitor (Christensen and Macleod 1945), E.A.C.A. (Alkjaersig et al. 1959a) and Trasylol (Steichele and Herschlein 1961).

B. PHYSIOLOGICAL CONSIDERATIONS

1. IN VIVO FIBRINOLYSIS

Investigations of the fibrinolytic enzyme system in vivo have posed many difficult problems, not least of these being the very real deficiencies in methodology. Also perplexing has been the problem of reconciling the known protean actions of plasmin with the necessary specificity for fibrinolysis in vivo. Furthermore, the appearance of an activator in plasma would produce a complex and continuously changing state resulting from simultaneous reactions involving activation, inhibition and the digestion of multiple susceptible proteins. Since under normal physiological conditions anti-plasmin inhibitor is present in considerable excess (as compared to available plasmin), it is considered unlikely that the fibrinolytic process in man is controlled by the level of free plasmin in the circulation: a view now supported by substantial evidence (Sherry et al. 1959b, Alkjaersig et al. 1959b),

Sawyer et al. 1960).

Rapid strides were made in understanding how this enzyme system worked in vivo, following the work of Fearnley (1953, 1961) and Sherry et al. (1959b), when it was considered that plasminogen was deposited in significant amounts whenever a clot formed, that plasminogen activator diffused into the clot and that the resultant generation of plasmin within this fibrin meshwork could be considered independent of the circulating plasma and thus protected from the large quantities of circulating anti-plasmins. This feature endows considerable specificity to the fibrinolytic enzyme system, and allows activation of small amounts of plasminogen within the interstices of the thrombus, thus resulting in extensive and protracted clot dissolution without other manifestations of systemic proteolysis.

Two important factors are essential to this so-called Sherry hypothesis: sufficient intra-clot plasminogen for subsequent activation to plasmin, and the passage of activator into the thrombus. Gross (1963), using isotopically labelled streptokinase, has demonstrated rapid and deep penetration into the thrombus. The problem of the plasminogen content of clots, however, is now more controversial. Sherry's group (Sawyer et al. 1961) observed that approximately 30% of the available plasminogen was adsorbed onto the fibrin during coagulation. Neither Ogston et al. (1966) nor Hedner et al. (1966)

have been able to confirm this observation, but the former group consider plasminogen diffuses into the clot following coagulation.

An entirely different hypothesis for in vivo clot lysis has been propounded by Ambrus and Markus (1960). They envisaged a circulating plasmin-anti-plasmin complex which provides a potent and readily available store of plasmin. In the presence of fibrin the complex is split and so releases the plasmin which is preferentially attracted to fibrin. More recently Olesen (1965) has combined the mechanisms of both theories and envisaged an activator-anti-activator complex which can be split at local sites of fibrin deposition.

It is possible that these different hypotheses relating to the mechanisms of physiological in vivo thrombolysis may not prove to be mutually exclusive. Whatever the final outcome, the Sherry hypothesis is now almost universally accepted as a good working hypothesis and thus under physiological circumstances thrombolysis would appear to be regulated, at least in part, by the availability of plasminogen activator.

2. SOURCE OF CIRCULATING PLASMINOGEN ACTIVATOR

Nolf (1904) first suggested that blood vessel walls might be the source of fibrinolytic enzymes. More recent observations by Kwaan and McFadzean (1956, 1957), Todd

(1959), Messer et al. (1962), Chakrabarti et al. (1963), Kwaan and Astrup (1963) and Neri Serneri et al. (1965) have confirmed, beyond reasonable doubt, that at least one source of circulating plasminogen activator arises from the intimal surface of veins, venules and capillaries. Whether tissue activator makes any contribution is more controversial, although Maki et al. (1965) have suggested mechanisms which would make this feasible.

3. CONTROL OF THE LEVEL OF CIRCULATING PLASMINOGEN ACTIVATOR

Observations by many investigators have indicated that the in vivo fibrinolytic mechanism appears to be continuously active and quite dynamic in its response to stimuli (Sherry et al. 1959b, Fearnley 1961, von Kaulla 1963). The plasma of healthy adults normally contains significant but small amounts of activator, but this can be rapidly and strikingly increased by a variety of physiological and pharmacological stimuli.

Despite the wide diversity of stimulating agents it is possible that the release of circulating plasminogen activator may be physiologically controlled by neurogenic and/or humoral mechanisms. Kwaan and McFadzean (1956, 1957) first postulated a neurogenic effector control of the intimal units. This work has been somewhat overlooked as it was based upon an incorrect assumption that

venous occlusion had no effect on fibrinolytic activity (Clarke et al. 1960, Tighe and Swan 1963). Despite these criticisms, recent investigators have again emphasised the possibility of a neurogenic and/or humoral control of plasminogen activator release (Schneck and Von Kaulla 1961) and Benetato et al. (1964) have provided interesting evidence to support the hypothesis of a specific hypothalamic area which controls the level of circulating plasminogen activator.

The final common mechanism for all these diverse stimuli may prove to be adrenaline. Holemans (1965) considered that the increased plasminogen activator following all known fibrinolytic stimulating procedures was secondary to vasoactive changes, so that resting areas of the vascular bed were washed out, or that intimal units were damaged following either anoxia (vasoconstriction) or stretching (vasodilatation).

Equally important for the control of blood levels of plasminogen activator are those for the subsequent removal of this substance. It is now reasonably certain that the liver plays an important role in the removal of at least some of circulating plasminogen activator (Fletcher et al. 1964, Januszko et al. 1966).

4. FIBRINOLYSIS - COAGULATION EQUILIBRIUM

In 1908, Nolf proposed the existence of a dynamic

equilibrium between fibrin deposition (coagulation) and fibrin removal (fibrinolysis). This hypothesis received the support of Copley (1954), Astrup (1956), Jensen (1956), Witte (1958), Roos (1958), Fearnley (1961), Salmon (1961) and Woolf (1961) and is envisaged in its fullest concept as a continuous process, in which the endothelial lining of vessel walls is coated with a thin layer of fibrin. Iatridis and Ferguson (1962, 1966) have postulated that the bridge between the two systems is Hageman Factor.

Despite the evidence in favour of this hypothesis, there is a considerable quantity of equally conflicting information (Gajewski and Alexander 1963, Kamel et al. 1963, Lewis 1963, Izak 1965, Lewis and Szeto 1965, Izak and Gajewski 1966). It is not proposed to examine the pros and cons for this hypothesis for, at present, it is impossible to either prove or disprove (Hjort and Hasselback 1961). However, even if the main argument in favour of this hypothesis is its obvious beauty, it has resulted in a large amount of research endeavour to which the contribution outlined in this thesis must be added.

C. PATHOLOGICAL CONSIDERATIONS

1. FIBRINOLYSIS AND OCCLUSIVE VASCULAR DISEASE

Rokitansky, 100 years ago, was the first to propose

that deposits of fibrin on the arterial intimal wall caused atherosclerosis. More recently this hypothesis has been revived by Duguid (1949, 1955) whose observations that fibrin can be deposited on arterial intima, covered by endothelium and thus incorporated into the vessel wall, have been partially confirmed by Harrison (1948), Hend (1949), Crawford and Levene (1952) and Ambrus et al. (1958). These findings have been extended by Astrup (1956) to relate them to the proposed coagulation - fibrinolysis equilibrium so that excessive coagulation or diminished fibrinolysis would result in excess intimal fibrin which is later incorporated into the vessel wall.

If intravascular fibrin formation plays a role in the development of atheroma, it follows that factors operating to remove it are of major importance. The subsequent efforts of various groups of investigators to demonstrate defective fibrinolysis in occlusive vascular disease has proved to be disappointing and controversial (Hume 1958, Nestel 1959, Lackner and Merskey 1960, Merskey et al. 1960, Nestel 1960, Goldrick 1961, Ogston 1962, Fearnley et al. 1963, Katz et al. 1963, Naimi et al. 1963, MacKay and Hume 1964). In a review of the recent literature, Fearnley (1965) has concluded that there is little evidence of a difference in blood fibrinolytic activity between males with overt coronary artery disease and aged matched controls, but there is some reason to suspect that defective fibrinolysis is present in post-menopausal women,

in women with ischaemic heart disease and in both sexes suffering from peripheral atherosclerosis.

2. FIBRINOLYSIS AND CANCER

The interesting possibility that the spread of malignant tumours, both directly and metastatically, may be governed by the ability of these cells to form and maintain fibrin has been postulated by Wood (1958) and O'Meara (1958). Cliffton (1966) has reported that the induction of an active fibrinolytic state, in animals given large doses of intravenous cancer cells, markedly reduced the number of metastases even when large numbers of tumour cells were shown to be present in the circulating blood. The significance of these findings remains, as yet, unresolved, but Thornes (1966) has recently emphasised that an important factor in the spread of cancer may be the ability of the cancer cell to maintain its fibrin in the face of the opposing fibrinolysis in the host. Should this hypothesis be confirmed then the activity of the fibrinolytic system of the host may be a factor of major significance. Although Thornes (1966) has concentrated on the individual variation in the production of antiplasmins it may prove that the ability to generate plasminogen activator is of equal importance.

3. FIBRINOLYSIS AND SHOCK

Innes and Sevitt (1964) have shown that during the first few hours following severe injury there is a marked acceleration of coagulation and fibrinolysis, followed by a fall to normal, but in some cases a fall well below normal in fibrinolysis. Hardaway (1966) has postulated that in many types of shock an important cause of a fatal outcome may be the widespread thrombosis of the micro-circulation, thus leading to severe and irreversible tissue anoxia. In the light of this hypothesis Hardaway has emphasised that situations of danger may arise if the endogenous fibrinolytic response is either too little or too late.

It is clear from the foregoing brief discussion of the possible role of the fibrinolytic enzyme system in occlusive vascular disease, spread of cancer and shock that the studies are grossly incomplete. Further useful information is unlikely to be obtained without either prolonged and carefully controlled investigations or an entirely fresh approach to the study of the physiological control mechanisms of fibrinolysis.

CHAPTER 2

HYPOTHESIS AND AIMS OF PROJECT

It is possible that some of the present uncertainty concerning the role of the fibrinolytic enzyme system in physiological and pathological situations arises from the known fact that the resting level of blood fibrinolysis can differ considerably between apparently healthy individuals and may fluctuate in any one person, from day to day, and throughout the same day (Fearnley 1959, Blix 1961). Most of the reported studies have been based upon single observations, and, in this situation, unless extreme and prolonged differences in fibrinolysis exist between the disease states under investigation and the normal controls, comparison by single estimations might fail to yield satisfactory results.

Certain physiological situations such as exercise (Biggs et al. 1947, Truelove 1951, Fearnley and Lackner 1955, Sherry et al. 1959*b*, Billimoria et al. 1959, Sawyer et al. 1960, Ogston and Fullerton 1961, Iatridis and Ferguson 1963, Jange et al. 1964, Ogston and McAndrew 1964, Burt et al. 1964) and mental anxiety (Macht 1952, Dreyfuss 1956, Friedman et al. 1958, Ogston et al. 1962) stimulate an increase in both coagulation and fibrinolysis (the latter being due to plasminogen activator). Careful analysis of the exercise studies reveals that some of the

subjects responded poorly. Those who have commented on these poor responders have given tentative explanations. Biggs et al. (1947) considered physical fitness relevant, Sawyer et al. (1960) inappropriate timing of sample collection, whereas Iatridis and Ferguson (1963) suggested that a failure to show the usual reaction to stress may have occurred. In fact, it is impossible to make any satisfactory conclusions from these earlier studies because of the variations in fibrinolytic assay techniques and the types of exercise used in different laboratories. Of no less importance is the fact that no attempt was made to ascertain whether this phenomenon was reproducible.

It is proposed that the accumulation of single sample absolute values of plasma fibrinolysis may be of limited value, particularly in a system which is assumed to be in a state of dynamic flux, and that attention should be turned to the capacity of the organism to produce a fibrinolytic response to stressful situations. Thus, evidence, if obtained, of a poor ability to generate plasminogen activator to stress may be of more physiopathological significance than the measurement of absolute resting values.

The following chapters describe the methods and results of a series of studies designed to investigate whether in a group of 50 apparently healthy young and middle aged human subjects there exists a small proportion whose mechanisms for generating plasminogen activator to stress are defective.

CHAPTER 3

METHODS I

SUBJECTS AND EXPERIMENTAL DESIGN

SUBJECTS

Two groups of volunteers were studied: 50 young healthy subjects (25 male and 25 female) aged between 18-30 years (mean age: 22.3 years) and 50 healthy middle-aged subjects (25 male and 25 female) aged between 42-57 years (mean age: 49.6 years).

All subjects were required to give a full medical history and in addition the middle-aged subjects received a medical examination which included:-

(1) Clinical examination of heart, peripheral pulses, chest and abdomen.

(2) Measurement of resting (supine 15 minutes) blood pressure (by auscultation).

(3) Urine analysis for sugar and albumin.

(4) Resting and post-exercise (2 minutes step-up test) electrocardiography.

All experiments were performed between 9.00 a.m. - 12.00 noon after a light breakfast, in a procedure room at 19-20° C. Subjects were requested to abstain from

smoking and excessive exercise on the morning of the experiment and, on arrival at the laboratory, were required to rest, lying down, for 30 minutes.

All subjects undertook the fibrinolytic stimulating procedures on more than one occasion. Most experiments were repeated at weekly intervals, but a few at intervals of several months. Nine of the young women, with a history of regular and trouble-free menstrual cycles, were studied at weekly intervals throughout a menstrual cycle.

EXERCISE

Previous investigations on the effect of exercise on fibrinolysis were not designed for a critical analysis of the ability of subjects to generate plasminogen activator. In the first instance some exercise procedures, by their very nature, were impossible to standardise, such as basketball (Sherry et al. 1959a, Sawyer et al. 1960), walking over moors (Ogston and Fullerton 1961) and running up and down stairs (Macfarlane and Biggs 1947). Other work in which exercise to exhaustion was used (Iatridis and Ferguson 1963, Burt et al. 1964) must be criticised because of fundamental problems of motivation and the possible danger for older subjects.

Truett et al. (1966) have recently stressed the practicability of submaximal exercise testing in

epidemiological studies of coronary heart disease. The type of the submaximal used in this study was based upon the work of Åstrand (1964). He emphasised certain points, all of which were considered:-

(a) Great muscle groups should be engaged in the test work, thus avoiding local muscular fatigue being a limiting factor.

(b) The work must be technically fairly easy so that mechanical efficiency is kept relatively constant, and the efficiency fairly high.

(c) The work load must be reproducible. (Apparatus which best satisfy (b) and (c) are the treadmill and bicycle ergometer.)

(d) Investigations should be made during a steady state, i.e. four to five minutes or longer after the start of the work.

(e) The work intensity should not be so high as to make motivation play a dominating part.

(f) The work intensity should not be too low otherwise psychological factors may influence the results.

The standard exercise procedure chosen was 8 minutes on a treadmill at 3.4 m.p.h. with a 5° elevation. Calculating from the normogram of Workman and Armstrong (1964) for predicting treadmill walking oxygen consumption, the energy expenditure for this exercise was of the order of 5K Cals/min., which has been classified by Christensen (1953) as moderate exercise.

A further attempt at improving reproducibility of the exercise was made by requesting each subject to adjust his stride to 120 paces/min. with the assistance of a metronome.

For the study of fibrinolytic reactivity to exercise, the procedure was as follows: immediately following the initial venepuncture, taken while at rest from a vein in the cubital fossa, each subject was exercised. At the termination of the 8 minute period the treadmill was stopped, the subject requested to sit down and the post-exercise blood sample withdrawn from a cubital vein in the other arm.

ADRENALINE INFUSIONS

Biggs et al. (1947) first demonstrated the ability of adrenaline to stimulate an in vivo increase in plasma fibrinolytic activity in man. This observation has been confirmed by others, including Truelove (1951), Kwaan et al. (1957), Sherry et al. (1959), Genton et al. (1961), Doni et al. (1963) and Neri Serneri et al. (1965).

Most of the previous studies on adrenaline induced fibrinolysis have used the subcutaneous route of administration. The many variable factors known to govern adsorption of subcutaneously administered drugs (Goodman and Gilman 1965) precluded this route on the grounds of inadequate reproducibility. For this reason, and those of

safety, the intravenous route was used in the study of generalised fibrinolytic reactivity to adrenaline.

Preliminary experiments, which will be discussed in a subsequent chapter, demonstrated that a satisfactory fibrinolytic response to intravenous adrenaline could be achieved with a dose of the order of 10 μgm./min. for 2 minutes. A correction factor was applied to give a more standardised dose by relating it to the body surface area. Thus the precise dose administered, using a Harvard Constant Infusion Pump, was 10 μgm./1.95 m^2 surface area/min. for 2 minutes in a total volume of 20 ml. normal saline.

The preparation of adrenaline used was that of Parke-Davis (batch no. 35-11-1-16: each ml. contained 1 mg. adrenaline as the hydrochloride dissolved in normal saline, with 0.5% chlorbutol as a preservative). This adrenaline preparation was kept at +4° C. and further diluted in normal saline immediately before use. No further preservative was added to the diluted adrenaline.

For the routine study of fibrinolytic reactivity to intravenous adrenaline the procedure was as follows: immediately following the 30 minute rest period, a 21 G. needle was inserted into a cubital vein, without local anaesthesia, a blood sample withdrawn (the "before sample"), the syringe detached and a connection made to the 50 ml. syringe in the Harvard Constant Infusion Pump by means of an Anaesthesia Extension Set (Baxter: BR - 335). The time from the withdrawal of the blood sample to the onset

of adrenaline infusion was 1 minute. At the end of the infusion period the anaesthesia extension set was clamped and 2 minutes after the end of the infusion a second blood sample was withdrawn (the "after sample") from a cubital vein in the opposite arm.

Electrocardiographic recordings were made before and during the adrenaline infusion. The development of any form of arrhythmia was regarded as an absolute indication to stop the infusion immediately: on no occasion was this necessary. Subjects were carefully briefed as to the possible subjective symptoms arising during the adrenaline infusion, and should they be unduly alarmed, then the infusion would be terminated forthwith: on no occasion was this necessary.

CHAPTER 4

METHODS II

MATERIALS AND ASSAY TECHNIQUES

*MATERIALS

Anticoagulant

Sodium citrate B.P. 3.8% (supplied by Boots Pure Drug Co. Ltd.).

Buffers

(a) Barbiturate buffer. Modified veronal buffer of pH 7.5 and ionic strength 0.15 (Owren, 1947).

(b) Tris buffer. 0.15 M solution of tris-(hydroxy-methyl)-amino-methane (Koch-Light Laboratories Ltd., Batch No. 11342) adjusted to pH 7.8.

(c) Phosphate buffer. 0.1 M at pH 7.6.

*(Full details of composition of the various reagents used in the assay techniques described are presented in the Appendix.)

Fibrinogen

A Blomback and Blomback (1957) preparation of human fibrinogen with a clottability of 97% (supplied by Kabi Pharmaceutical Company, Batch No. 83164) was used. Aliquots of a 1.5 g./100 ml. solution of fibrinogen in tris buffer were stored in plastic tubes at -20° C. After thawing it was diluted to 0.15 g./100 ml. in tris buffer and used for fibrin plates.

Thrombin

Topical thrombin (Parke Davis and Co., Batch No. 03179A) was used. A solution of 50 units/ml. in tris buffer was prepared for fibrin plates, one of 5 units/ml. in barbiturate buffer for euglobulin lysis time estimations, and one of 100 units/ml. in physiological saline for fibrinogen estimations. Aliquots of all these solutions were stored at -20° C. in plastic tubes and used immediately after thawing.

Streptokinase

The preparation produced by Lederle Laboratories (Batch No. 2201-66) was used for plasminogen assays. It was diluted to 2,000 units/ml. in phosphate buffer and aliquots stored in plastic tubes at -20° C.

Urokinase

The standard reference preparation (2,400 Plough units) produced by Leo Pharmaceuticals, Denmark (Batch No. 63062) was used. Solutions of 3 units/ml. in tris buffer were prepared and aliquots stored at -20° C. in plastic tubes.

Casein

The preparation produced by L. Light and Co. Ltd. was used (Batch No. 5051).

Glassware

All glassware was siliconised by means of a 3% solution of I.C.I. M550 silicone in trichlorethylene.

Petri dishes

Plastic petri dishes with an internal diameter 8.8 cm. (supplied by Staynes Laboratories) were used. Each petri dish was discarded after use.

Glass beads

0.15 mm. diameter: supplied by the English Glass Company, Leicester.

ASSAY TECHNIQUES (excluding euglobulin lysis time assay)

Fibrinogen assay

A modification of the original method of Ratnoff and Menzie (1951) was used. In this assay the fibrinogen is clotted with thrombin and left overnight to ensure complete coagulation in a medium containing sufficient E.A.C.A. to prevent further proteolysis. The tyrosine released from the fibrin by hydrolysis with sodium hydroxide is estimated with Folin Ciocalteu's reagent. A standard proportion of tyrosine in the fibrinogen/fibrin molecule is assumed.

All assays were performed in triplicate in unsiliconised 5" x 5/8" test tubes. Reagents were added in this order: 6.0 ml. 0.85% saline-E.A.C.A. solution, 0.1 ml. thrombin solution (100 units/ml. in 0.85% saline), 0.2 ml. 2.5% calcium chloride and 0.2 ml. of test substance (plasma or euglobulin solution). The contents were mixed by shaking and left to clot overnight at + 4° C. After overnight clotting approximately 0.5 ml. of glass beads (diameter 0.15 mm.) was added, the tube shaken and centrifuged at 3,000 r.p.m. for 2 minutes. The supernatant was sucked off and discarded, an equivalent volume of 0.85% saline added, the tube shaken and centrifuged as before. This washing procedure with saline was repeated three times. After the last removal of the saline, 0.4 ml. of

10% sodium hydroxide was added and the solution boiled in a water-bath for 20 minutes. The tube was then cooled under tap water and 0.6 ml. 5% trichloracetic acid added, followed by 2 ml. 0.5N sodium hydroxide and 0.6 ml. (1 : 2 diluted) Folin Ciocalteu's reagent. The contents were mixed and 15 minutes later the colour intensity was read at 650 mμ against a reagent blank on a Unicam S.P.600. The fibrinogen concentration was calculated from a standard tyrosine curve and these readings were converted to fibrinogen by multiplying by 11.7. The mean difference between triplicate observations over a wide range of fibrinogen levels, expressed as a percentage of the mean of these observations, was 6.2% $\pm$ 0.45.

Plasminogen assay

The caseinolytic assay of Remmert and Cohen (1949) as modified by Alkjaersig et al. (1959b) was used. Anti-plasmin was first destroyed by incubating the plasma with acid. The acid is neutralised with alkali and buffer and streptokinase is then added to convert the plasminogen to plasmin, which is assayed by caseinolytic assay: the amount of tyrosine released from the casein being a measure of the amount of plasmin present.

All assays were performed in triplicate in 5" x 5/8" unsiliconised test-tubes. 0.5 ml. of test substance (plasma or euglobulin solution) was added to 0.5 ml. N/6

HCl and left at room temperature for 15 minutes. 0.5 ml. N/6 NaOH was then added, followed by 1 ml. phosphate buffer (0.1M), and 0.5 ml. streptokinase (2,000 units/ml. in phosphate buffer, 0.1M). After mixing, 2 ml. were withdrawn and transferred to a test-tube containing 2 ml. 10% trichloracetic acid. The remaining 3 ml. in the original test-tube were incubated for 60 minutes at 37° C. in a water-bath and the 3 ml. 10% trichloracetic acid were added. The pre- and post-incubation samples were then centrifuged at 3,000 r.p.m. for 2 minutes and the supernatant filtered by gravity through a Whatman's No. 1 filter paper. 1 ml. aliquots of the respective filtrates were then transferred to a test-tube containing 5 ml. 0.5N NaOH and 1.5 ml. 5% trichloracetic acid. To this mixture 1.5 ml. Folin Ciocalteu's reagent (diluted 1 : 2) were added. After 15 minutes at room temperature the optical density was read on a Unicam S.P.600 at 650 mμ, reading the pre-incubation sample against the 60 minutes incubation sample. The milligrams of tyrosine released were calculated from a standard curve, and one casein unit equalled 180 μg. tyrosine released in one hour. The mean percentage error over a wide range of normal values was 2.5% ± 0.31.

Fibrin plates

Unheated human fibrin plates were prepared by a

modification of the method of Astrup and Mullertz (1952). (This was regarded as a semi-quantitative measure of plasminogen activator). 10 ml. of 0.15G% fibrinogen in tris buffer were pipetted into a plastic petri dish and clotted immediately with 0.2 ml. thrombin solution on a level table. Twenty minutes after the addition of thrombin, to ensure complete clotting, 0.03 ml. samples of the test material, in triplicate, were dropped onto the fibrin films. The plates were incubated at 37° C. for 24 hours and the products of the perpendicular diameters taken as a measure of fibrinolytic activity. Sensitivity of the plates prepared each day was checked by observing the area of lysis after drops of urokinase (3.0 units/ml.) were added. The mean percentage error of this method was 12.4% $\pm$ 0.61.

E.A.C.A. human fibrin plates were used in some studies for plasmin assay (McNicol and Douglas 1964). Standard human fibrin plates were prepared but before clotting 1 ml. of E.A.C.A. (10^{-2}M) in tris buffer was added and thoroughly mixed.

Urokinase inhibitor assay

Urokinase inhibitor assays were based on the method of Blix (1964). The test material was incorporated in the fibrin film and the degree of inhibition compared against the control. Human fibrin plates were prepared

as above, but before clotting 1 ml. of euglobulin solution, diluted in saline (0.85%), was added and thoroughly mixed. A series of four plates was used containing dilutions of $\frac{1}{10}$, $\frac{1}{25}$, $\frac{1}{50}$ and a control with 1 ml. saline. 0.03 ml. drops of urokinase (3.0 units/ml. in tris buffer) were then placed onto the fibrin film.

CHAPTER 5

METHODS III

EUGLOBULIN LYSIS TIME ASSAY

Hedin (1904) first demonstrated the presence of a weak proteolytic enzyme in serum by precipitation of the globulins. This observation was later extended by Milstone (1941) and Macfarlane and Pilling (1946) and this technique is now generally regarded as a sensitive and useful one for the assay of uninhibited plasma plasminogen activator (Sawyer et al. 1960, Iatridis and Ferguson 1963, Fletcher et al. 1964, Moser and Hajjar 1966). By suitable dilution of the plasma and adjustment of pH using diluted acetic acid (Milstone 1941) or CO_2 (Von Kaulla and Schultz 1958) a euglobulin precipitate is formed, the contents of which include plasminogen activator, plasminogen and fibrinogen. The precipitate is collected, removed from the supernatant (which contains the anti-fibrinolytic substances (Blix 1964)), resuspended in a buffer and clotted with thrombin. The time required for complete lysis, from the addition of thrombin, is taken as the euglobulin lysis time. The time is inversely related to the level of plasminogen activator.

Although this assay procedure is basically simple to

execute it is fraught with theoretical and practical difficulties (Blix 1961). In view of this fact, and that it represented a central part in the assessment of fibrinolytic reactivity in the subsequent studies, it was felt necessary to carry out certain studies in an effort to obtain a satisfactory standardised procedure.

There are four major factors which have been regarded as influencing the euglobulin lysis time: substrate variability, blood withdrawal and subsequent handling, and euglobulin fraction preparation and end-point recording.

Substrate Variability

The major components in the euglobulin fraction, which are of interest from the fibrinolytic point of view, are plasminogen activator, plasminogen and fibrinogen. An apparent disadvantage of this method is that these substrates are supplied in this clot lysis system from the subject under study and therefore normal variations in plasma plasminogen and fibrinogen may affect the results to an unknown extent (Kowalski et al. 1959, Blix 1961, Fearnley 1965).

This problem was studied by measuring the euglobulin lysis time and the euglobulin content of plasminogen and fibrinogen in a series of 33 subjects. Fig. 2 shows a plot of the results and it is apparent that there is no

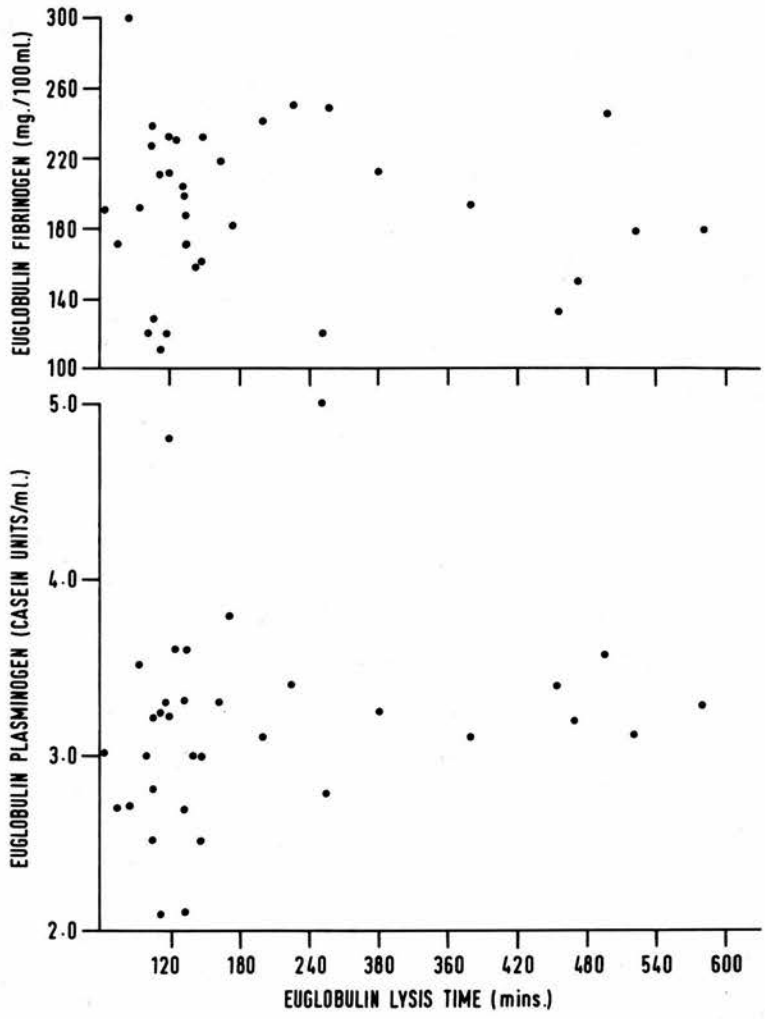

FIG.2 EUGLOBULIN PLASMINOGEN AND FIBRINOGEN PLOTTED AGAINST THE EUGLOBULIN LYSIS TIME.

correlation between the individual substrates and the euglobulin lysis time. These results would seem to indicate that the euglobulin lysis time is a valid measurement of plasminogen activator and are in agreement with the results and conclusions of Fletcher et al. (1964) and Moser and Hajjar (1966).

Blood Withdrawal and Subsequent Handling

(a) Venous occlusion

Venous occlusion has been demonstrated to increase the blood fibrinolytic activity in the occluded arm (Clarke et al. 1960, Holemans 1963, Iatridis et al. 1966), and Ata et al. (1964) have stressed the importance of the strictest attention being paid to the venepuncture technique in fibrinolytic investigations. The work of Tighe and Swan (1963), however, would suggest that as much as 2 minutes occlusion is required to demonstrate this effect. On the basis of these findings venous occlusion was permitted for no longer than 30 seconds for all studies reported in this thesis.

(b) Deterioration effects due to delayed assay procedures

Loss of fibrinolytic activity, of withdrawn blood, due to delayed assay procedures was first stressed by Fearnley et al. (1952) and Truelove (1953) and more recently by Januszko et al. (1965). A small series of

experiments was therefore undertaken to assess the practical limit of this problem.

45 ml. of fresh blood were added to 5 ml. pre-cooled anticoagulant, thoroughly mixed and placed in a bath of melting ice. 5 ml. aliquots were poured into a pre-cooled siliconised centrifuge tube at regular intervals and immediately centrifuged for subsequent assay of the euglobulin lysis time. The results are shown in Fig. 3 and clearly confirm the previous work, but do show that little or no deterioration occurs if the blood is processed within 15 minutes of withdrawal. Accordingly, in all subsequent experiments blood was centrifuged within 15 minutes of withdrawal.

(c) Low temperature blood handling

Fig. 3 also confirms the findings of Fearnley et al. (1952) and Truelove (1953) that the stability of blood plasminogen activator is enhanced by a low temperature technique. Accordingly, all procedures, once the blood samples were withdrawn, were performed at +4° C.

(d) Glass contact effects

According to Flute (1960) an anti-activator, which is precipitated in the euglobulin fraction, is generated when blood comes into contact with glass. No attempt has been made to confirm this observation but all glassware used was thoroughly siliconised (full details of the technique

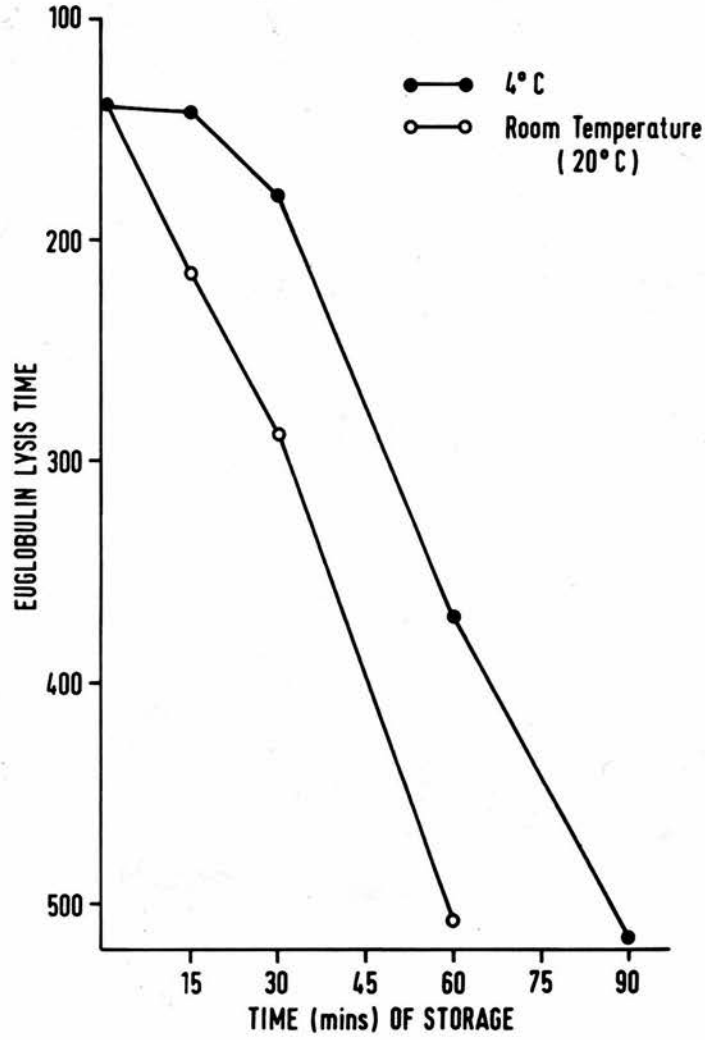

Fig.3. Deterioration effects on the euglobulin lysis time of blood left standing at +4°C and room temperature (+20°C) (mean of 5 experiments)

are recorded in the Appendix).

(e) Centrifugation

There is evidence that platelets contain anti-plasmin (Alkjaersig 1961, Holemans and Gross 1961) and proactivator (Greig and Cornelius 1961) activity. In order to avoid this variable source of error euglobulin fraction was precipitated from platelet poor plasma according to Iatridis and Ferguson (1963).

Euglobulin Fraction Preparation

In an excellent study of the problems of euglobulin precipitation, Blix (1961) clearly demonstrated that a most critical factor affecting the fibrinolytic activity of the euglobulin fraction was the pH at which it was precipitated. The following experiments were performed in order to confirm and extend these findings, to assess their significance to the euglobulin lysis time method as applied to the subsequent studies on fibrinolytic reactivity and to adjust the method accordingly.

Euglobulin fractions were precipitated by adding plasma from one subject to dilute acetic acid, in proportions of 1 : 9 respectively. The dilute acetic acid concentrations were so arranged that the final pH range varied from 5.0 - 6.4. Assays on the resultant euglobulin fractions were made of the euglobulin lysis times,

area of lysis on standard human fibrin plates and E.A.C.A.-fibrin plates, plasminogen, fibrinogen and inhibitor content. The results are arranged in Table 1 and confirm the findings of Astrup and Rasmussen (1958) and Blix (1961) that maximum yield of plasminogen activator, without spontaneous autocatalytic activation of plasminogen, is obtained at a pH of approximately 6.0. The results also indicate that any variation in the pH of euglobulin precipitation will affect the resultant euglobulin lysis time.

In the light of these findings further studies were made to determine whether using fixed volumes of dilute acid provided satisfactorily reproducible pH levels between individuals. Triplicate samples of euglobulin suspensions were made from fresh plasma samples obtained, almost simultaneously, from 15 healthy subjects. The pH of these suspensions was then determined using a Beckman Expanded Scale pH Meter.

The results, shown in Table 2, demonstrate that the range of final pH was between 5.58 - 5.78. Part of this variation may be due to the variability in the buffering power of plasma (Salenius 1957). The importance of this finding is that individual variations in the euglobulin lysis time, using pre-set quantities of dilute acetic acid, may be partially due to variation in the pH at which the euglobulin fraction is precipitated. Subsequent studies on fibrinolytic reactivity therefore took these results

TABLE 1

THE EFFECTS OF PRECIPITATING THE EUGLOBULIN FRACTION AT DIFFERENT pHs

Subject No.	Precipitation pH	Euglobulin Lysis Time (mins.)	Area of Lysis (mm^2) on Standard Human Fibrin Plates	Area of Lysis (mm^2) on E.A.C.A. Fibrin Plates	Euglobulin Fibrinogen (mg./100 ml.)	Euglobulin Plasminogen (c.u./ml.)	Plasma Fibrinogen (mg./100 ml.)	Plasma Plasminogen (c.u./ml.)	Inhibitor Assay (Area in $m.m.^2$)			
									Euglobulin Dilution $\frac{1}{10}$	$\frac{1}{25}$	$\frac{1}{50}$	Saline Control
1	5.0	18	350	0	214	3.89	(440)	(4.31)	440	410	414	(400)
	5.2	242	340	0	264	4.24						
	5.6	226	342	0	262	4.28						
	6.0	193	380	0	244	4.18			424	400	422	
	6.4	114	484	49	192	3.76						
2	5.0	84	504	0	172	2.94	(364)	(3.25)	396	396	384	(388)
	5.2	117	484	0	194	3.46						
	5.6	108	496	0	188	3.49						
	6.0	73	566	0	168	3.30			396	388	384	
	6.4	44	614	25	148	2.04						
3	5.0	620	225	0	106	4.05	(262)	(5.44)	424	424	416	(416)
	5.2	820	210	0	152	5.21						
	5.6	734	210	0	178	5.18						
	6.0	615	232	0	149	5.00			424	424	410	
	6.4	300	294	25	102	4.96						
4	5.0	345	314	0	85	3.92	(202)	(4.29)	400	400	400	(410)
	5.2	450	248	0	117	4.24						
	5.6	380	278	0	160	4.28						
	6.0	240	348	0	140	4.14			410	414	400	
	6.4	175	398	49	130	3.98						

TABLE 2

THE pH OF EUGLOBULIN SUSPENSIONS OF 15 HEALTHY SUBJECTS

Subj. No.	pH of triplicate samples			Mean pH
1	5.66	5.64	5.66	5.65
2	5.73	5.70	5.73	5.73
3	5.69	5.69	5.69	5.69
4	5.65	5.64	5.64	5.64
5	5.66	5.67	5.68	5.67
6	5.64	5.64	5.66	5.64
7	5.75	5.75	5.79	5.78
8	5.60	5.63	5.61	5.62
9	5.64	5.62	5.64	5.63
10	5.66	5.67	5.68	5.67
11	5.63	5.63	5.64	5.63
12	5.59	5.68	5.64	5.60
13	5.60	5.62	5.68	5.63
14	5.59	5.58	5.58	5.58
15	5.68	5.69	5.69	5.68

Range 5.58 - 5.78

Euglobulin was precipitated from 1 ml. of plasma in 19 mls. of distilled water to which 0.18 ml. of 1% acetic acid had previously been added.

into account by precipitating all euglobulin fractions, by careful titration, to pH 6.0. Because of this critical pH control, dilute acetic acid was preferred to the CO_2 method of Von Kaulla and Schultz (1958).

Comment

Although the purpose of these studies was not an exhaustive examination of euglobulin preparation, sufficient information was obtained which answered the questions originally posed. Accordingly, the following techniques for the euglobulin lysis time were undertaken and rigidly adhered to during the experiments described in subsequent sections.

1. Blood sampling and centrifugation

9 ml. of blood were withdrawn by clean venepuncture, with the minimum of venous occlusion (less than 30 seconds), into a siliconised syringe. The blood was immediately transferred to a previously cooled centrifuge tube, housed in a receptacle of melting ice, containing 1 ml. of anticoagulant. The centrifuge tube was covered with parafilm, inverted twice and returned to the melting ice where it remained until the second sample was obtained. Following mixing, both were centrifuged at 3,400 r.p.m. (2,000 g.) at +4° C. for 20 minutes. Immediately after, the upper third of the plasma was removed, using a siliconised

pasteur pipette, into a pre-cooled plastic tube. Subsequent handling of the plasma, such as euglobulin precipitation and placing plasma drops onto unheated fibrin plates, was done immediately.

2. Euglobulin lysis time assay

1 ml. of plasma was transferred to a siliconised centrifuge tube in melting ice, containing 19 ml. of distilled water and 0.18 ml. of 1% acetic acid. The pH was finally adjusted to 6.0, on a Beckman Zeromatic pH meter, 0.25% acetic acid being used. Precipitation time was 10 minutes from the addition of the plasma and was done in melting ice. The euglobulin suspensions were then centrifuged at 3,400 r.p.m. for 20 minutes at +4° C. The supernatant was discarded, the inside of the centrifuge tube wiped dry with a tissue, and the precipitate resuspended in 1 ml. of barbiturate buffer pH 7.4. 0.24 ml. of this euglobulin solution was transferred, in triplicate, to 3" by 3/8" (7.5cm by 1 cm.) siliconised test-tubes and 0.24 ml. of thrombin solution (5 units/ml. in barbiturate buffer pH 7.4) added immediately. The test-tubes were placed in a water-bath at 37° C. and the time taken from the addition of thrombin to complete lysis was recorded as the euglobulin lysis time.

End-point Recording : The Development of a 12 Channel Automatic Euglobulin Clot Lysis Recorder

An important source of error in the euglobulin lysis time is the determination of the end-point of clot lysis, which is normally done visually (Baumgarten et al. 1960). This subjective assessment of clot lysis has proved to be inadequate on the grounds of reproducible accuracy objectivity and the uneconomic use of the laboratory worker's time. A fully automatic device capable of determining the lysis end-point of 12 euglobulin clots simultaneously, with good reproducibility and accuracy, will now be described. This machine has been developed and built under the guidance of the author by members of staff of the Edinburgh University Department of Medical Physics, and has been used in all the studies on fibrinolytic reactivity in the middle-aged group of volunteers and most of the physiological studies related to adrenaline infusions.

(1) Principles of operation

The operational principles of this machine are not new and follow the basic concepts of other photoelectric methods of recording coagulation and clot lysis (Nygaard 1941, Harrower 1962, Kuhnke and Tettenborn 1963, Nanninga et al. 1964, Newman 1964). Light is shone through an opaque clot and the amount transmitted is recorded on a photoelectric cell, the output of which is recorded on a

direct-writing recorder. As clot lysis progresses increased light passes to the photoelectric cell until no further change occurs when lysis is completed. The euglobulin lysis time is taken as the time interval from the addition to the point on the chart when no further change takes place.

(2) Design

(a) Lysis Chamber (Castle), Fig. 4a, 4b and 4c

The Castle comprises a solid copper block (thermal conductivity 0.918 CAL/sq. cm./° C.) into which holes were machined to accommodate the various components and light paths. A heating element, housed in the block, maintained the temperature at 37° C. by means of a temperature sensing and controlling system. Insulation was found to be unnecessary: standing on a bench at room temperature (20° C.) its temperature was maintained at 37° C. with a differential of only 0.2° C. A common light source was used to illuminate all sample tubes, the photocells being located radially about this source. The provision of a single light source for 12 clots assured electrical uniformity of the 12 channels.

(b) Control unit, Fig. 5

Each photocell was fed to its appropriate bridge network and a "set position" control, using a meter in each

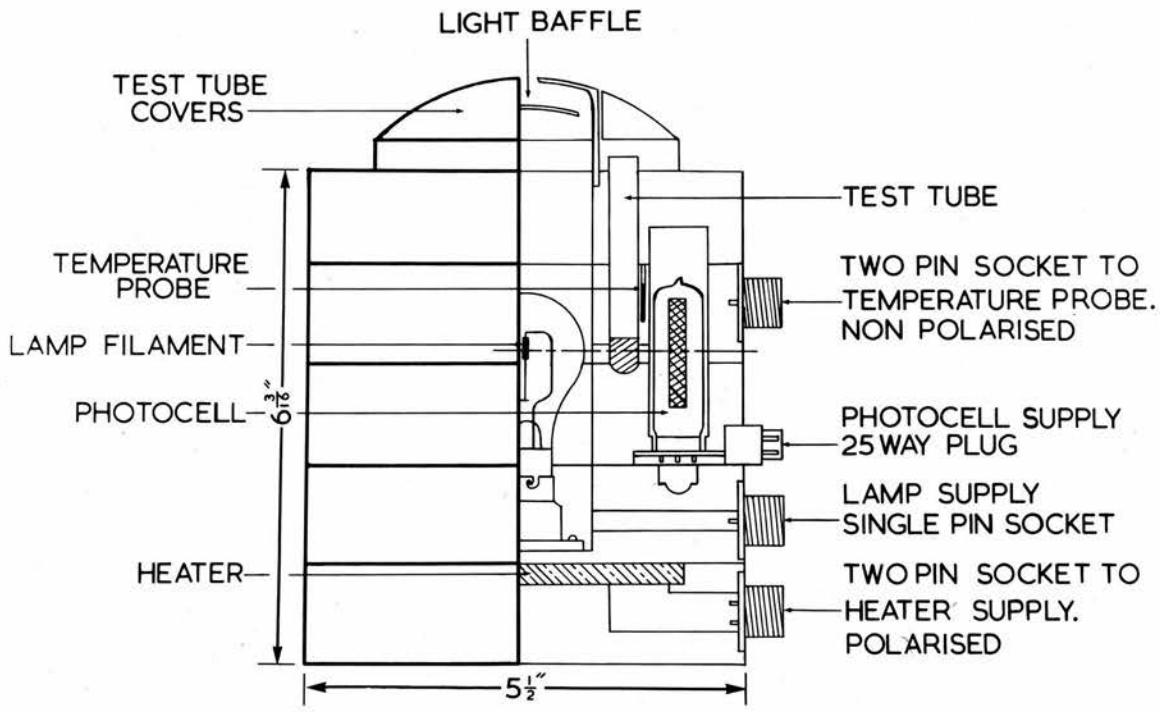

Fig. 4a. A diagrammatic representation of the copper Castle.

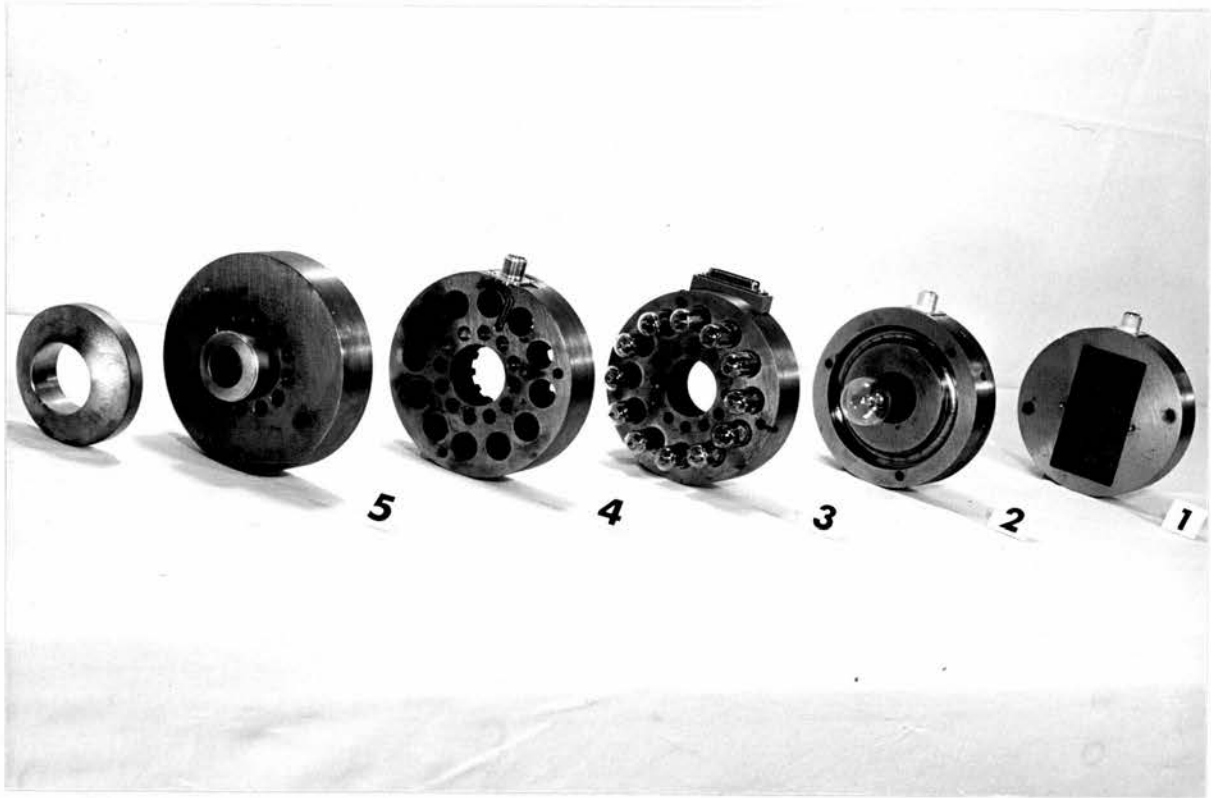

Fig. 4b. The components of the copper Castle viewed from above.

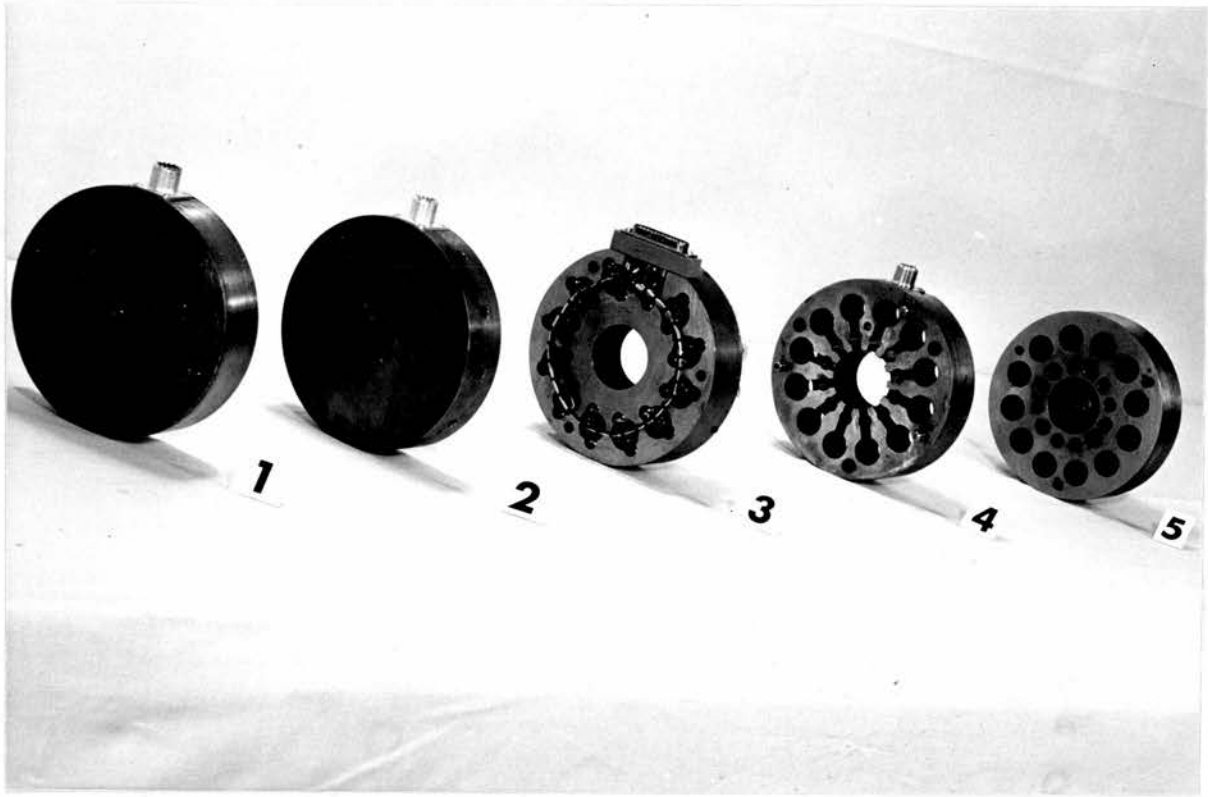

Fig. 4c. The components of the copper Castle viewed from below.

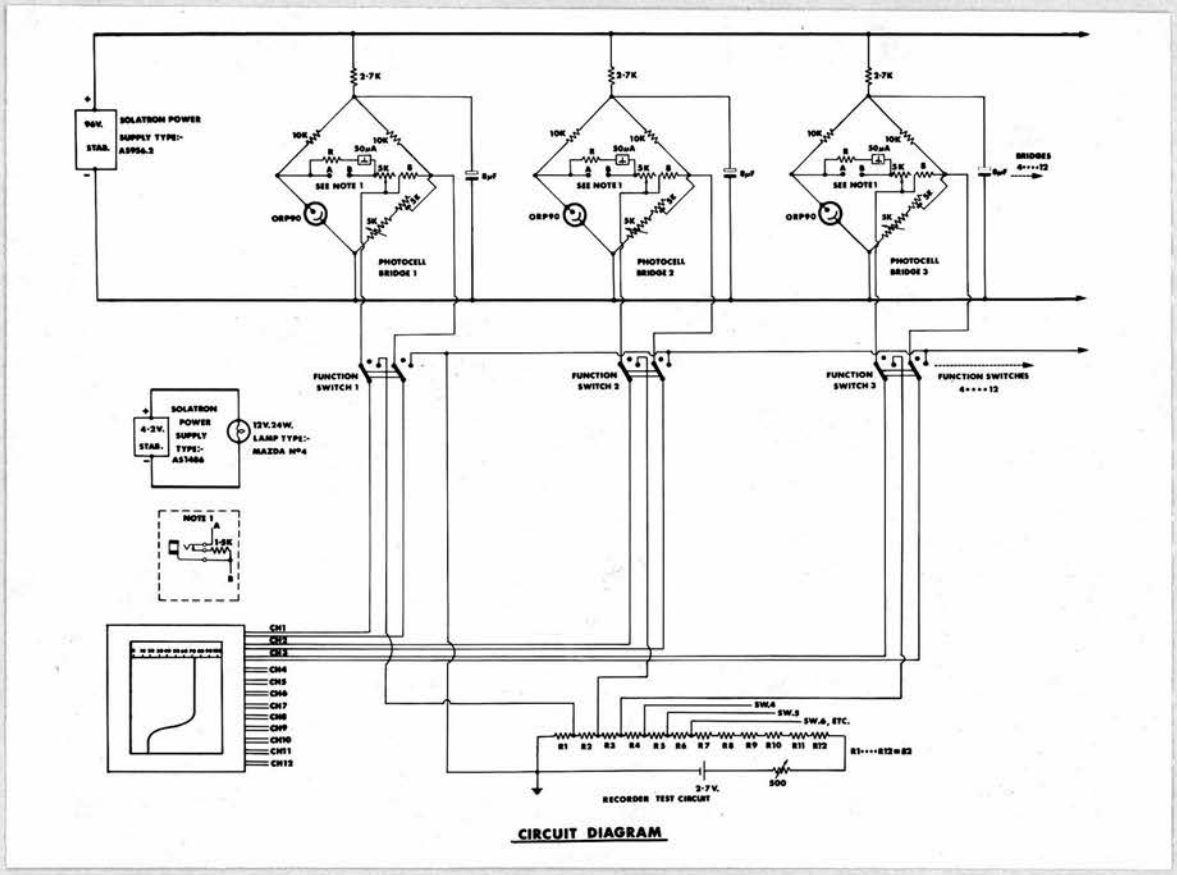

Fig. 5. A circuit diagram of the control unit.

bridge, enabled the trace for each clot to be adjusted to any required position on the chart paper. This arrangement greatly facilitated easy operation of the apparatus and permitted its use by a technician after no more than 5 minutes' instruction.

(c) Recorder

The outputs from the bridges were coupled to a Leeds and Northrup 'Cleretrend' 12 point recorder, provision being made for matching the photocell outputs. The recorder selected each channel in turn and printed a dot corresponding to the deflection from that channel. With a paper speed of 3" per hour the resultant trace is a continuous line. Channel identification is by colour and number.

A photograph of the complete assembly is shown in Fig. 6.

(3) Operational characteristics

Fig. 7 is a typical example of an actual chart record of the lysis of 2 sets of 6 identical clots and shows the good reproducibility between channels. (The clots were transferred from a water-bath at 37° C. to the Castle 80 minutes after the addition of thrombin.) Table 3 records the lysis times of a series of clots, obtained by the visual and automatic methods. These results were recorded

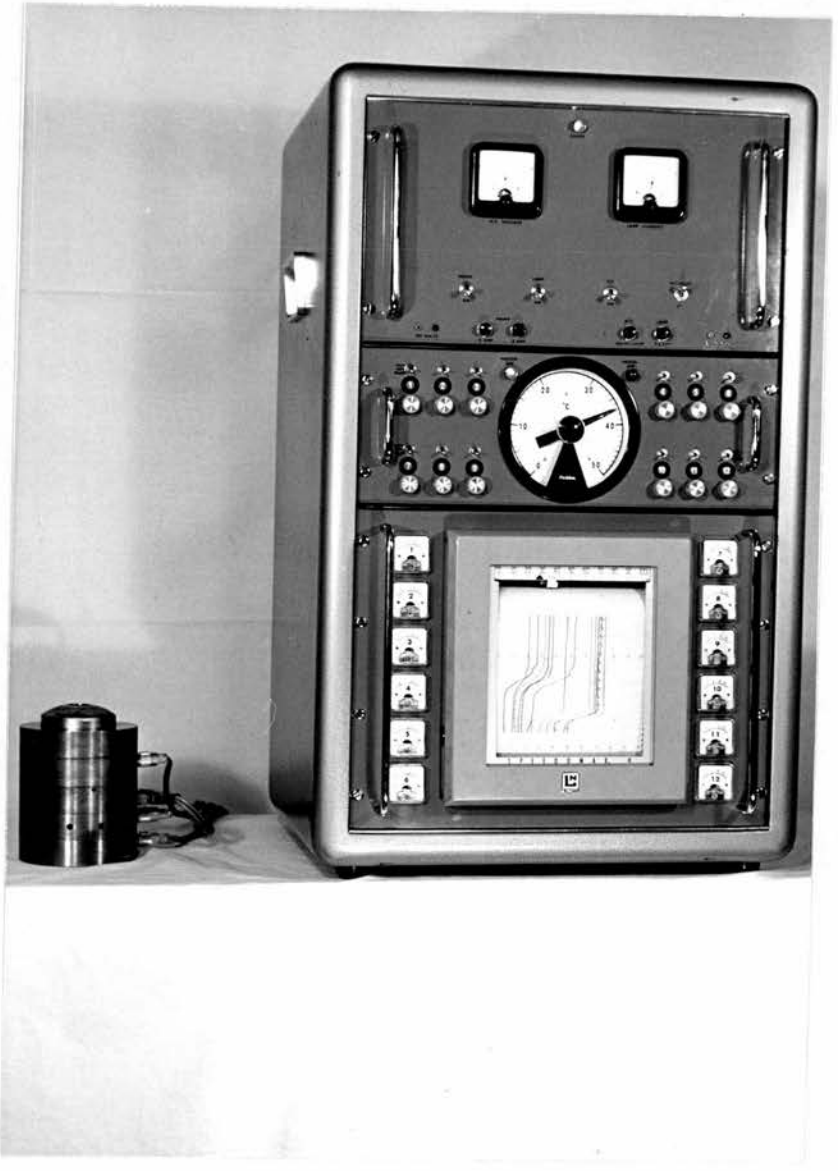

Fig. 6. The complete assembly.

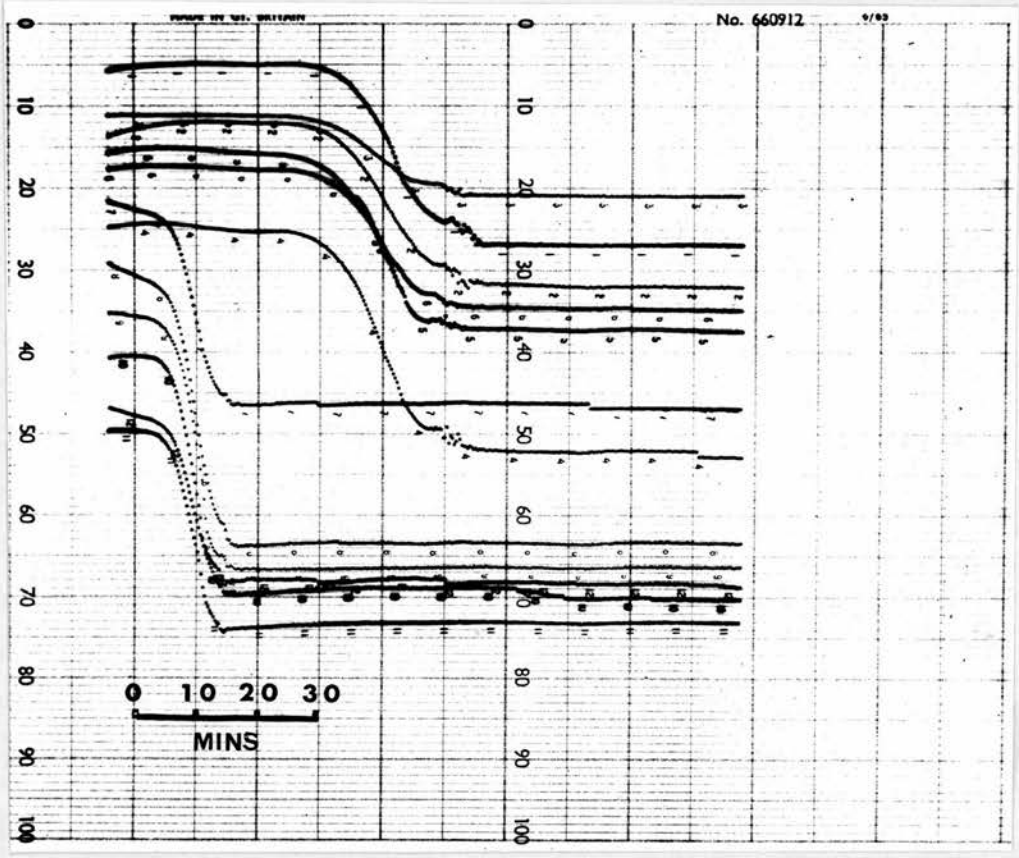

Fig. 7. A photographic reproduction of an actual chart record of the lysis of 2 sets of 6 identical clots.

TABLE 3

COMPARISONS OF THE EUGLOBULIN LYSIS TIMES RECORDED VISUALLY AND BY THE AUTOMATIC CLOT LYSIS RECORDER

Sample No.	Mean Euglobulin Lysis Time (mins.)	
	Visual Method	Automatic Recorder
1	224	233
2	87	90
3	124	120
4	68	67
5	624	642
6	104	103
7	220	233
8	154	150
9	197	196
10	324	331

in a blind fashion: the operator observing the clot lysis visually was not allowed to see the clot lysis recorder, and the clot lysis charts were read by an independent observer. There was no statistically significant difference between both sets of readings ($0.3 \geq p < 0.4$).

Comment

It is believed that this machine represents an important advance in the field of fibrinolysis research, for it has achieved a degree of accuracy, reliability and objectivity seldom realised by the visual methods. Its large capacity, which could be greatly increased, makes it an attractive instrument for those engaged in population studies and others requiring serial samples. Facilities are also available for the output from any channel to be recorded on other instruments. This means that it can be readily adapted for other clot lysis systems and those studies related to automatic coagulation end-point recording.

The collective reproducibility of the visual method among four different workers in the laboratory, expressed as a mean percentage error over a wide range of normal values, where the euglobulin clot preparation was performed by one person, as described in the previous section, was 10.5% $\pm$ 1.2. The mean percentage error for the automatic device was 2.4% $\pm$ 0.5. This difference was statistically significant ($p < 0.05$).

CHAPTER 6

RESULTS I

RESTING AND MODERATE EXERCISE STUDIES IN 50 YOUNG SUBJECTS

1. Resting levels of euglobulin lysis time

Table 4 shows the results of all experiments. The resting level of circulating plasminogen activator, as measured by the euglobulin lysis time, varied from subject to subject (range 68-502 min., mean: 183 min., standard deviation 100 min.) and from day to day in some subjects. No urokinase inhibitor content was demonstrated in the euglobulin solutions studied (50 samples taken over a wide range of euglobulin lysis times). The female group had a significantly higher mean level of plasma plasminogen activator than the male group ($0.001 > p < 0.01$), but there were no consistent changes related to the phases of the menstrual cycle in the 9 women studied.

Comment

The finding that, even under carefully standardised assay procedures, the resting euglobulin lysis times varied from subject to subject and from day to day in some

TABLE 4

EUGLOBULIN LYSIS TIME BEFORE AND AFTER MODERATE EXERCISE
IN MALE AND FEMALE SUBJECTS AGED 18 TO 30 YEARS

MALES					FEMALES				
Subj. No.	Euglobulin Lysis Time		% Increase	Mean % Increase	Subj. No.	Euglobulin Lysis Time		% Increase	Mean % Increase
	Before	After				Before	After		
+M 1	140	71	49		F 1	156	71	54	
	660	332	50	49		228	100	56	54
	500	257	49			198	95	52	
M 2	240	110	54		F 2	112	70	38	
	304	136	55	55		113	75	34	37
						*99	62	37	
						97	61	37	
M 3	195	120	48		F 3	115	55	50	
	105	58	45			88	45	49	48
	135	80	40	43		177	96	46	
	77	45	42			*140	74	47	
	83	51	39						
	148	92	38						
M 4	620	440	29		F 4	117	49	58	
	84	60	29			425	117	73	65
	270	200	26	29		120	45	63	
	318	213	33						
	210	156	26						
M 5	61	35	43		F 5	160	83	48	
	75	42	44			200	100	50	49
	57	35	39	37					
	65	45	31						
	81	50	38						
M 6	115	70	39		F 6	222	97	56	
	146	96	34	37		135	71	47	
						204	90	56	52
						*192	95	51	
						193	97	50	
						143	70	51	
M 7	105	60	43		F 7	102	48	53	
	131	78	41			141	65	54	52
	77	47	39	39		90	45	50	
	76	50	34						

+Cigarette smokers (10/day)

*Results during menstruation

TABLE 4 - continued

MALES					FEMALES				
Subj. No.	Euglobulin Lysis Time		% Increase	Mean % Increase	Subj. No.	Euglobulin Lysis Time		% Increase	Mean % Increase
	Before	After				Before	After		
+M 8	550	440	20	24	F 8	132	53	60	64
	211	172	19			370	134	64	
	745	505	32			300	100	67	
M 9	143	60	58	58	F 9	132	57	57	53
	150	65	57			*135	62	54	
	150	62	59			117	50	47	
						95	45	53	
M 10	105	63	40	40	F 10	84	43	49	48
	95	59	38			77	42	45	
	103	60	42			80	41	49	
+M 11	255	124	51	51	F 11	123	72	42	42
	315	153	51			131	75	43	
	262	129	51			*132	74	44	
						120	75	38	
+M 12	120	95	21	26	F 12	132	62	53	51
	159	116	27			*135	65	52	
	272	192	29			145	71	51	
	151	106	30			142	62	49	
	245	185	24						
	132	98	26						
+M 13	494	211	57	52	+F 13	180	92	49	52
	720	370	48			306	142	54	
	274	137	50			250	117	53	
						148	71	52	
M 14	100	54	46	50	F 14	595	380	36	39
	113	56	50			185	110	41	
	110	52	53			90	53	41	
M 15	112	52	54	54	F 15	105	45	57	51
	223	104	53			110	55	50	
	180	80	56			100	50	50	
						*105	55	48	
M 16	257	143	44	38	F 16	115	95	48	43
	120	80	33			138	81	41	
	140	90	36			171	101	41	

+Cigarette smokers (10/day)

*Results during menstruation

TABLE 4 - continued

MALES					FEMALES				
Subj. No.	Euglobulin Lysis Time		% Increase	Mean % Increase	Subj. No.	Euglobulin Lysis Time		% Increase	Mean % Increase
	Before	After				Before	After		
M 17	72	65	10	10	F 17	150	76	49	47
	108	86	20			198	93	51	
	200	184	8			*178	87	45	
	320	310	3			178	95	41	
+M 18	189	99	48	49	F 18	166	85	49	44
	270	137	49			176	104	41	
						162	95	42	
M 19	65	51	22	29	F 19	176	103	42	40
	84	56	33			202	124	39	
	94	64	32			200	120	40	
+M 20	250	160	36	36	F 20	121	68	43	44
	230	150	35			131	73	44	
						117	65	44	
M 21	116	87	25	24	F 21	68	30	56	56
	108	82	24			80	33	59	
	110	85	23			*82	32	56	
						89	39	54	
M 22	120	75	38	39	F 22	90	45	50	50
	500	300	40			105	53	50	
						112	58	49	
M 23	174	89	49	44	F 23	115	60	48	46
	135	77	43			107	60	44	
	100	60	40						
M 24	539	324	40	40	F 24	99	52	47	45
	269	162	40			96	56	42	
						110	61	45	
M 25	107	63	42	40	F 25	118	73	38	38
	76	45	41			120	74	38	
	95	60	37						

+Cigarette smokers (10/day)

*Results during menstruation

subjects confirms the observations of Blix (1961). In view of the results, discussed previously (see Fig. 2), demonstrating the absence of a correlation of euglobulin substrate content and euglobulin lysis time and the absence of detectable anti-urokinase in euglobulin fractions, it was concluded that there was insufficient evidence to preclude the probability that individual differences in resting level of plasma plasminogen activator do in fact exist. Furthermore, it would appear that it may fluctuate widely in some individuals, as was suggested by Fearnley (1961). These findings once again emphasise the danger of over/simplification in the interpretation of fibrinolytic studies based on single estimations.

The observation of a statistically significant higher level of circulating plasminogen activator in the female group compared to the men is contrary to the findings of Beller et al. (1964) and Brakman et al. (1966). It is not possible, at present, to be certain of an explanation for this apparent discrepancy but two factors may prove to be relevant. Previous conclusions were reached, in the main, from single observations: the possible misinterpretation of such observations has already been discussed. Another probable reason for the marked sex difference in base lines in the present work may have been the heterogeneous nature of the two groups with respect to cigarette smoking. There is fragmentary evidence to indicate that cigarette smoking may have a deleterious effect on blood

fibrinolysis (Gibelli et al. 1964, Sogani and Joshi 1965). Although this feature was not the subject of any specific studies in this project, it was interesting to note that when the cigarette smokers (more than 10/day) were removed from both groups (6 males and 1 female), and the remaining 19 males and 24 females compared, there was no statistically significant difference in resting levels of plasminogen activator between these groups ($0.3 > p < 0.4$).

The finding of no consistent changes in the resting level of plasminogen activator during a normal menstrual cycle is contrary to the earlier observations of Smith and Smith (1945) and Willson and Munnell (1946) but in agreement with the more recent observations of Beller et al. (1964), Rao (1964), Brakman et al. (1966) and Menon (1966).

2. Exercise studies

(a) Pattern of fibrinolytic response

Fig. 8 shows the pattern of fibrinolytic response to the standard exercise procedure. Serial cubital venous blood samples were obtained by separate venepunctures before, during and after the exercise and the euglobulin lysis times recorded. The results demonstrated that the maximum fibrinolytic response occurred at the termination of the exercise. Based on these findings, subsequent routine studies on fibrinolytic reactivity to exercise were performed by simply comparing the euglobulin lysis

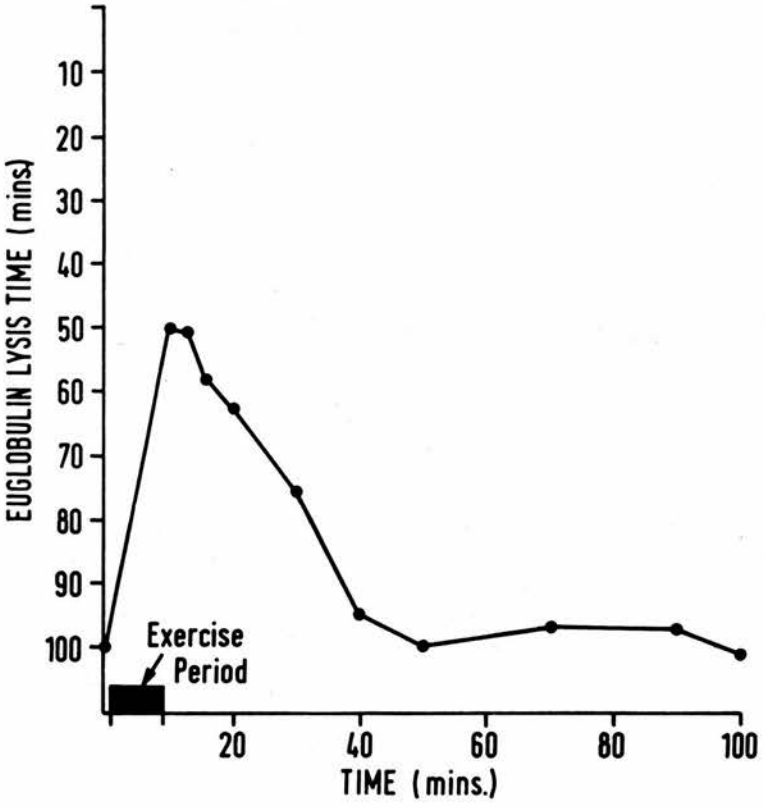

Fig. 8. The pattern of fibrinolytic response to the standard exercise procedure. (Mean of 10 subjects)

times of blood samples obtained immediately before and after the exercise.

(b) Fibrinolytic response in young subjects

If A and B represent the resting and post-exercise euglobulin lysis times, respectively, the percentage response following exercise was calculated as $A-B/_A \times 100$. The results of this calculation for each experiment and the mean percentage response for each subject are shown in Table 4. These results would indicate that for any one subject the fibrinolytic response was reproducible (correlation coefficient $r = 0.9929$ and $p < 0.001$). Furthermore, there was a highly significant individual variation in response (analysis of variance showed $F = 36.7$, which was significant at the 1% level). Fig. 9 demonstrates the frequency distribution of the percentage response in all subjects whereas Fig. 10 represents the distribution when the sexes are separated. Statistical analysis by the students 't' test showed a significantly higher level of response in the females compared to the males ($0.001 \geq p < 0.01$).

There appeared to be no consistent changes in the fibrinolytic response to the moderate exercise procedure throughout the menstrual cycle.

Comment

Despite the daily fluctuation in the resting

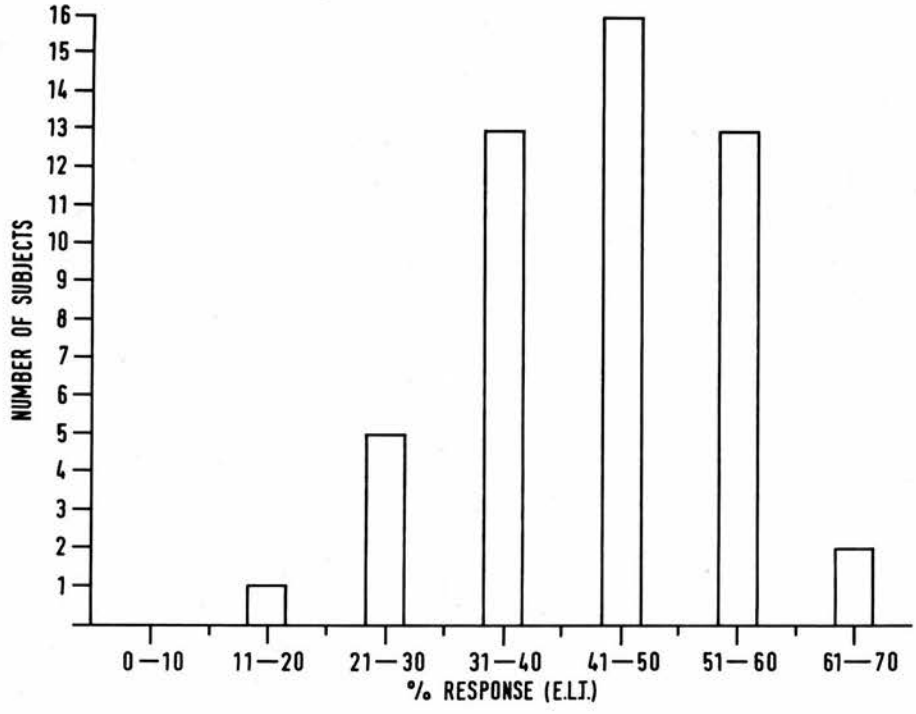

FIG.9. FREQUENCY DISTRIBUTION OF PERCENT RESPONSE TO MODERATE EXERCISE IN ALL SUBJECTS.

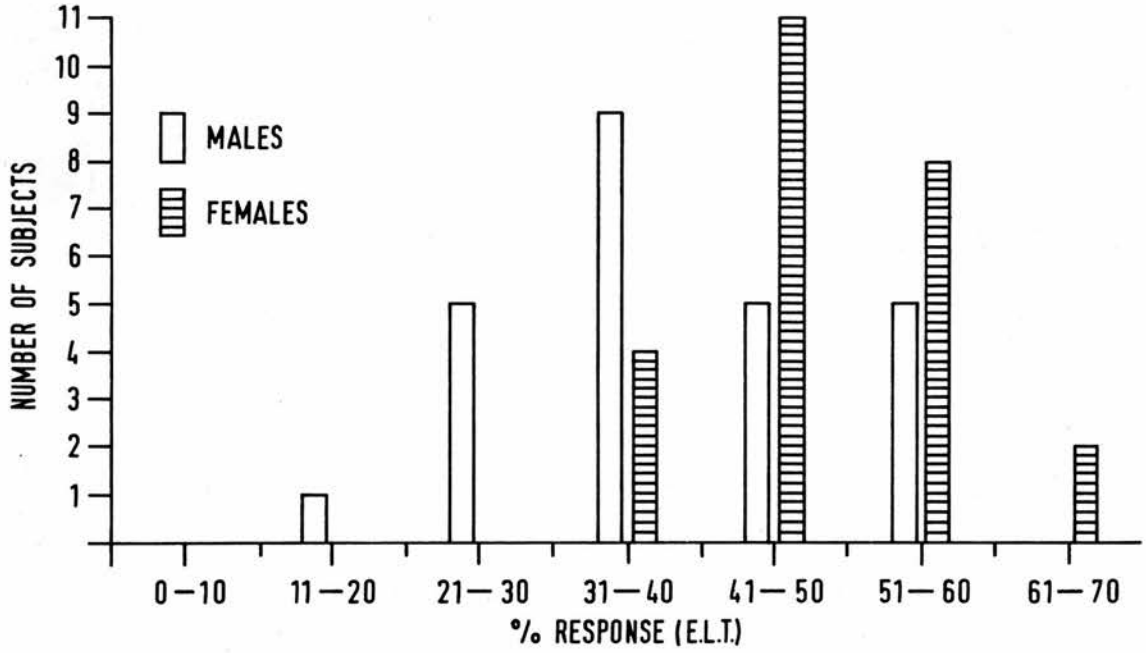

FIG.10. FREQUENCY DISTRIBUTION OF PERCENT RESPONSE TO MODERATE EXERCISE IN MALES AND FEMALES.

plasminogen activator level the fibrinolytic response, expressed as a percentage of the pre-exercise level, in normal young subjects was found to be a reproducible phenomenon. There appeared to be significant differences in response between individuals and it was possible to isolate a group of poor responders. Of no less interest was the finding that females of the same age-group had a greater fibrinolytic response than males, and that in the 25 females studied no poor responder was demonstrated. However, the fibrinolytic response was not influenced by the various phases of the menstrual cycle.

Although the fibrinolytic response to exercise is believed to be due to increased levels of circulating plasminogen activator (Sherry et al., 1959a, Iatridis and Ferguson 1963), and although euglobulin plasminogen and fibrinogen concentrations are not related to resting levels of the euglobulin lysis time, it is nevertheless possible that changes occurred in these substrates which could account for the individual variation in fibrinolytic responses to the standard exercise. This problem was studied in 21 subjects in whom euglobulin plasminogen and fibrinogen were estimated before and after the exercise. There was a consistent small increase in both substrates following exercise. However, when the percentage mean post-exercise increase in both these substrates was plotted against the percentage euglobulin lysis time response (Fig. 11), then no correlation was observed

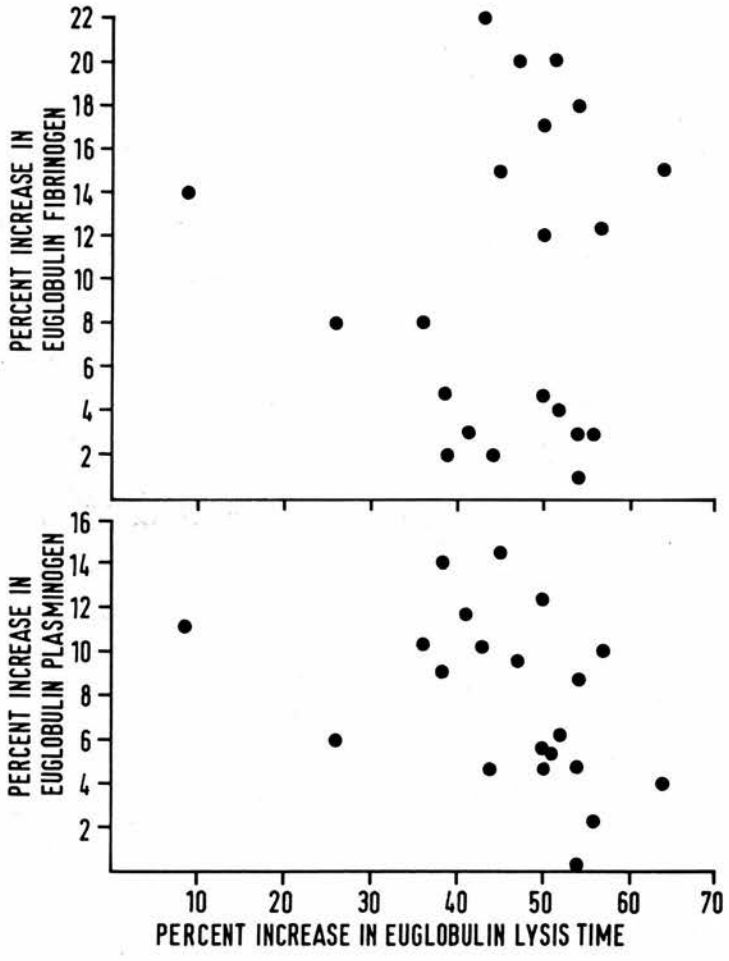

Fig.11. Percentage post-exercise increase in euglobulin substrates plotted against the percentage increase in the euglobulin lysis time.

($r = 0.024$: $0.1 \geq p \leq 0.2$ for fibrinogen and $r = 0.397$: $0.05 \geq p \leq 0.1$ for plasminogen).

It is possible that the variation in response between individuals may simply mirror the individual variation in stress to the exercise. If pulse rate response is a measure of the exercise stress, then the absence of a correlation between mean fibrinolytic response and mean pulse rate response in the 22 subjects studied (Fig. 12; $r = 0.112$: $p \geq 0.1$) would not support this view. When the two sexes were compared (Table 5), however, the position was less clear: the higher mean fibrinolytic response in the females was associated with a significantly higher mean pulse rate response (males 70/min., females 84/min., $p \leq 0.001$). This latter finding is in agreement with the work of Åstrand (1952), who considered that the cause of a higher female pulse rate response might be a stronger distaste in women for physical exertion, or that men might be more efficient working machines. Thus on the basis of these findings alone, the sex difference in fibrinolytic response in favour of the females could prove to be due to the increased effort in this group.

Severe exercise has been shown to increase body temperature variably in different individuals (Holmgren and McIlroy 1964) and severe heat stress increases plasma fibrinolytic activity (Bedrak et al. 1963). Although at present no data are available which might elucidate this possible explanation of the results reported here, it is

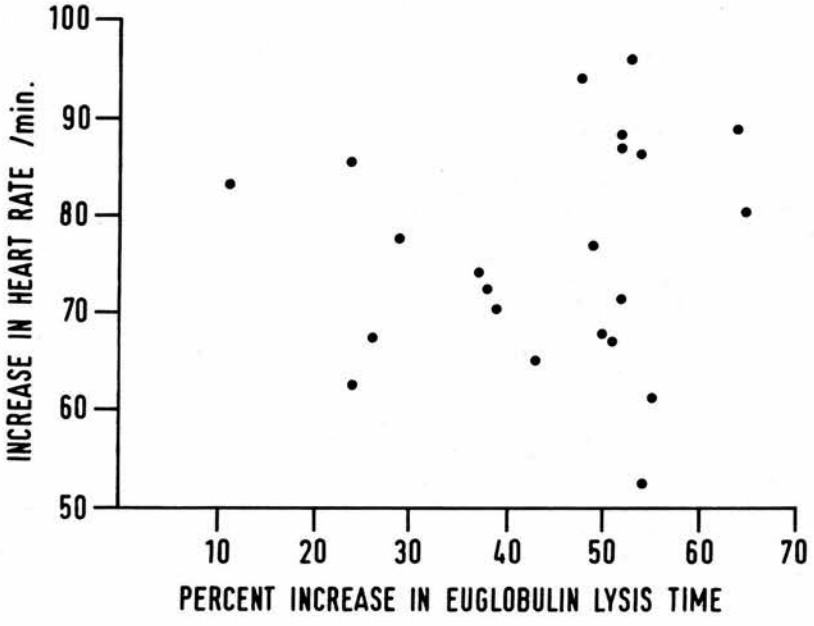

FIG.12 PERCENT INCREASE IN EUGLOBULIN LYSIS TIME PLOTTED AGAINST INCREASE IN HEART RATE, FOLLOWING MODERATE EXERCISE.

TABLE 5

THE MEAN PERCENT RESPONSE IN EUGLOBULIN LYSIS TIME AND PULSE RATE INCREASE FOLLOWING MODERATE EXERCISE IN 13 MALES AND 9 FEMALES

MALES				FEMALES			
Subj. No.	Mean % Response (E.L.T.)	PULSE RATES Before & After	Pulse Rate Response	Subj. No.	Mean % Response (E.L.T.)	PULSE RATES Before & After	Pulse Rate Response
M 1	49	75 - 152	+ 77	F 1	54	63 - 149	+ 86
M 2	55	59 - 120	+ 61	F 2	39	60 - 130	+ 70
M 3	43	85 - 150	+ 65	F 4	65	80 - 160	+ 80
M 4	29	71 - 148	+ 77	F 7	52	73 - 161	+ 88
M 5	37	62 - 136	+ 74	F 8	64	72 - 161	+ 89
M 8	24	83 - 148	+ 65	F 9	53	63 - 159	+ 96
M 12	26	71 - 138	+ 67	F 10	48	71 - 165	+ 94
M 13	52	71 - 142	+ 71	F 13	52	67 - 154	+ 87
M 14	50	69 - 136	+ 67	F 15	51	81 - 148	+ 67
M 15	54	72 - 124	+ 52				
M 16	38	84 - 156	+ 72				
M 17	14	60 - 143	+ 83				
M 21	24	51 - 136	+ 85				
			Mean = 70				Mean = 84

regarded as unlikely that the exercise procedure used in this work was sufficient to produce significant changes in core temperature which are assumed necessary to cause increased fibrinolysis.

It was not possible to explain the poor responders on the basis of physical fitness (Biggs et al. 1947). Four of the young subjects studied were highly trained athletes whose mean response ranged from 24 - 54%. Iatridis and Ferguson (1963) demonstrated the generation of a fibrinolytic inhibitor following exercise in one of their poor responders. Using the method of Blix (1964), no anti-urokinase content was demonstrated in any of the euglobulin fractions precipitated at pH 6.0.

Unlike the baseline studies, the sex difference in fibrinolytic response to exercise was not altered when the smokers were withdrawn.

CHAPTER 7

RESULTS II

RESTING LEVELS AND EXERCISE RESPONSES IN A GROUP OF 50 NORMAL MIDDLE-AGED SUBJECTS

1. Resting levels of plasminogen activator

The results in Table 6 were similar to those observed in the young subjects: the resting level of plasminogen activator, as measured by the euglobulin lysis time, varied from subject to subject (range 69-865 min., mean 232 min., standard deviation 191 min.) and from day to day in some subjects.

There was no statistically significant difference between the sexes in the middle-aged group ($0.1 \geq p \leq 0.2$) nor between the total young and middle-aged populations ($0.1 \geq p \leq 0.2$).

2. Exercise response studies

The fibrinolytic response was calculated as previously and the results of all experiments are shown in Table 6. There was good individual reproducibility of response ($r = 0.787 : p \leq 0.001$) and a highly significant variation

TABLE 6

EUGLOBULIN LYSIS TIMES BEFORE AND AFTER MODERATE EXERCISE
IN MIDDLE-AGED MALE AND FEMALE SUBJECTS

MALES					FEMALES				
Subj. No.	Euglobulin Lysis Time		% Increase	Mean % Increase	Subj. No.	Euglobulin Lysis Time		% Increase	Mean % Increase
	Before	After				Before	After		
+1	924	759	18	10	+*1	180	97	46	47
	989	999	0			159	77	52	
	747	689	8			148	83	44	
	800	690	14						
+2	220	101	54	49	*2	367	112	70	72
	160	110	31			262	69	74	
	239	116	52						
	324	139	57						
3	127	45	65	58	*3	105	37	65	62
	122	61	50			86	35	59	
	270	107	60						
4	118	71	40	36	*4	210	119	43	46
	107	70	35			127	65	49	
	102	68	33						
5	75	33	56	57	+5	95	45	53	51
	82	35	57			87	45	48	
+6	208	103	50	52	+6	118	48	59	66
	228	107	53			176	49	72	
7	120	78	35	30	7	220	83	62	50
	107	80	25			113	70	38	
						153	68	66	
						98	57	42	
8	159	79	50	48	*8	120	30	75	74
	95	52	45			235	65	72	
+9	123	50	59	52	9	90	55	39	32
	147	72	51			120	90	25	
	106	56	47			103	71	31	

+Cigarette smokers (10/day)

*Post-menopausal women

TABLE 6 - continued

MALES					FEMALES				
Subj. No.	Euglobulin Lysis Time		% Increase	Mean % Increase	Subj. No.	Euglobulin Lysis Time		% Increase	Mean % Increase
	Before	After				Before	After		
10	288 201	135 96	53 52	53	10	101 115 100	40 48 42	60 58 58	59
11	113 100	68 66	40 34	37	+11	233 152 225	90 62 75	61 59 67	62
+12	98 84 75 58	48 54 46 28	51 36 39 52	45	+*12	578 412 816 741	566 275 877 568	2 33 0 23	15
13	538 126	284 59	47 53	50	13	84 84 105	44 48 53	48 43 50	47
14	176 682	96 383	46 44	45	14	85 89 160	51 49 87	40 45 46	44
15	90 237	40 110	56 54	55	15	102 221 111 112	53 140 59 54	48 37 47 52	46
+16	93 95 101	60 51 56	36 46 45	42	+*16	200 212	73 81	64 62	63
17	641 765 840 700	301 660 602 351	33 14 28 50	44	*17	69 61	41 34	41 44	43
18	144 95	96 63	33 33		+*18	940	484	49	54
19	65 105	34 55	48 48	48	19	134 94	65 51	52 46	49

+Cigarette smokers (10/day)

*Post-menopausal women

TABLE 6 - continued

MALES					FEMALES				
Subj. No.	Euglobulin Lysis Time		% Increase	Mean % Increase	Subj. No.	Euglobulin Lysis Time		% Increase	Mean % Increase
	Before	After				Before	After		
20	127 90	77 65	39 28	34	*20	100 81	49 46	51 43	47
+21	717 707 668 462 428	420 559 411 312 296	41 21 39 33 31	33	+*21	79 72	39 39	51 46	49
22	253 151	108 59	57 61	59	*22	210 255	93 131	56 49	53
+23	513 642 624	445 542 625	13 16 0	10	23	120 94	62 54	48 43	46
24	78 62	38 34	51 45	48	*24	99 116	67 60	32 48	40
+25	124 123	73 75	41 39	40	25	128 121	34 45	73 63	68

+Cigarette smokers (10/day)

*Post-menopausal women

between individuals (analysis of variance showed F = 28.4, which was significant at the 1% level). There was a significantly higher response in the female group compared to the males ($0.02 \geq p \leq 0.05$), but no significant difference in the response between the middle-aged and young groups as a whole ($0.4 \geq p \leq 0.5$) (Fig. 13).

Comment

There are four important factors which mitigate against a satisfactory analysis of the results on the effect of age, sex and fibrinolysis: (1) the study was not linear and therefore cross-sectional in nature, (2) the numbers involved were relatively small and the measurements obtained had a high standard deviation, (3) only 13 of the middle-aged females were post-menopausal and even in this group the mean duration of the menopause was only 4.2 years, and (4) it was not possible to compensate for differences in energy expenditure for the same submaximal exercise procedure between the young and the middle-aged groups.

Previous studies on the effect of age and fibrinolysis are sparse and contradictory. Buckell and Elliot (1959) and Swan (1963) reported a diminished level of fibrinolysis with increasing age, whereas Hume (1961) observed an increase. No change was demonstrated by Sawyer et al. (1960), Fearnley et al. (1963) and Moser and

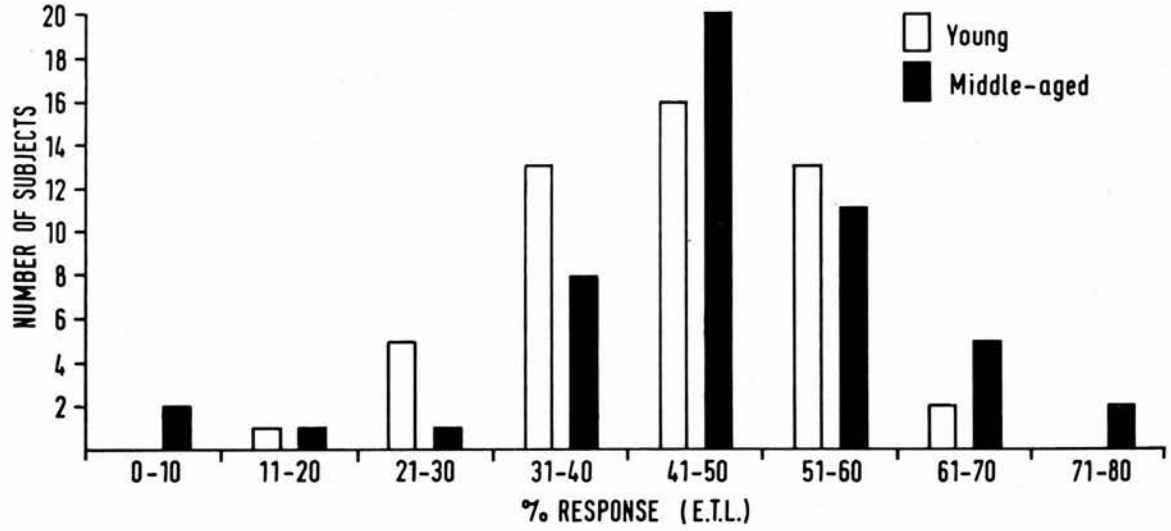

Fig. 13. Frequency distribution of percentage response to moderate exercise in young and middle-aged subjects.

Hajjar (1966), so that the results of this present study are in agreement with these authors' conclusions.

The absence of a sex difference in the euglobulin lysis times is in contrast to the findings in the younger group. This observation was not influenced when the post-menopausal women were excluded from the statistical analysis. It is possible that the presence of a higher proportion of cigarette smokers in the middle-aged female group ($^{8}/_{25}$) compared to the young female group ($^{1}/_{25}$) was an important factor. When the middle-aged non-smoking females were compared with the total middle-aged male group ($^{9}/_{25}$) smokers, then the difference was almost statistically significant ($0.05 \geq p \leq 0.1$); furthermore, the middle-aged smoking population had a significantly prolonged mean resting euglobulin lysis time compared to the non-smoking population ($0.01 \geq p \leq 0.02$). These results once again emphasise the fact that cigarette smoking may prove to have a deleterious effect on the resting level of circulating plasminogen activator, as measured by the euglobulin lysis time.

Within the middle-aged group the findings of the fibrinolytic response experiments were similar to those in the young group: good individual reproducibility, significant variation between individuals and the isolation of a group of poor responders. There also appeared to be a higher response in the females compared to the men ($0.02 \geq p \leq 0.05$) but this was less significant than the sex

difference observed in the young group ($0.001 > p < 0.01$). Although there was no significant difference in the mean responses of the pre- and post-menopausal middle-aged women, it was of interest to note that one of the middle-aged poor responders was a post-menopausal woman: no poor responders were demonstrated in the young female subjects. Despite these remarks no firm conclusions are yet possible, but there is at least suggestive evidence that the marked sex difference in fibrinolytic response to exercise in the young subjects may be diminished later in life. This type of shift was demonstrated in the pulse rate responses in this study (Table 7) and was also demonstrated by Becklake et al. (1965).

Evidence is available, from other sources, which shows that with increasing age there is a concomitant increase in energy expenditure for any fixed submaximal exercise procedure (Durnin and Mikulicic 1956, Shock 1964, Becklake et al. 1965, Hanson et al. 1966), and that this is masked by isolated studies on pulse rate responses (Robinson 1938, Åstrand 1958, Asmussen and Mathiasen 1962, Dill and Consolazio 1962, Hollman 1963, Strandell 1964). Fig. 14 shows the fibrinolytic response to various grades of exercise and demonstrates the increased fibrinolytic response with increasing severity of exercise. In view of the expected higher energy expenditure in the middle-aged subjects, the finding of no statistically significant difference in fibrinolytic response between the two age-

TABLE 7

PULSE RATE CHANGES IN MIDDLE-AGED SUBJECTS
FOLLOWING MODERATE EXERCISE PROCEDURE

MALES					FEMALES				
Subj. No.	Mean % Response (E.L.T.)	PULSE RATES			Subj. No.	Mean % Response (E.L.T.)	PULSE RATES		
		Before	After	Pulse Rate Response			Before	After	Pulse Rate Response
1	10	70	150	+ 80	1	47	69	126	+ 57
2	49	65	125	+ 60	2	72	71	150	+ 79
3	58	59	150	+ 91	3	62	70	152	+ 82
4	36	52	116	+ 64	4	46	79	150	+ 71
6	52	57	142	+ 85	5	51	74	157	+ 83
8	48	70	150	+ 80	6	66	76	140	+ 64
9	52	58	143	+ 85	7	50	75	150	+ 75
10	53	68	150	+ 82	8	74	65	150	+ 85
11	37	58	120	+ 62	9	32	82	148	+ 66
12	45	69	140	+ 71	10	59	72	145	+ 73
13	50	68	136	+ 68	11	62	75	159	+ 84
15	55	70	130	+ 60	12	15	84	154	+ 70
16	42	75	152	+ 77	13	47	90	152	+ 62
18	33	61	125	+ 64	14	44	70	150	+ 80
19	48	67	145	+ 78	15	46	93	158	+ 65
20	34	67	120	+ 53	16	63	75	143	+ 68
21	33	70	130	+ 60	17	43	71	151	+ 80
22	59	64	155	+ 91	18	54	79	164	+ 85
23	10	80	146	+ 66	19	49	-	-	-
24	48	58	138	+ 80	20	47	84	164	+ 80
25	40	65	150	+ 85	24	40	72	159	+ 87
				Mean = 74					Mean = 74

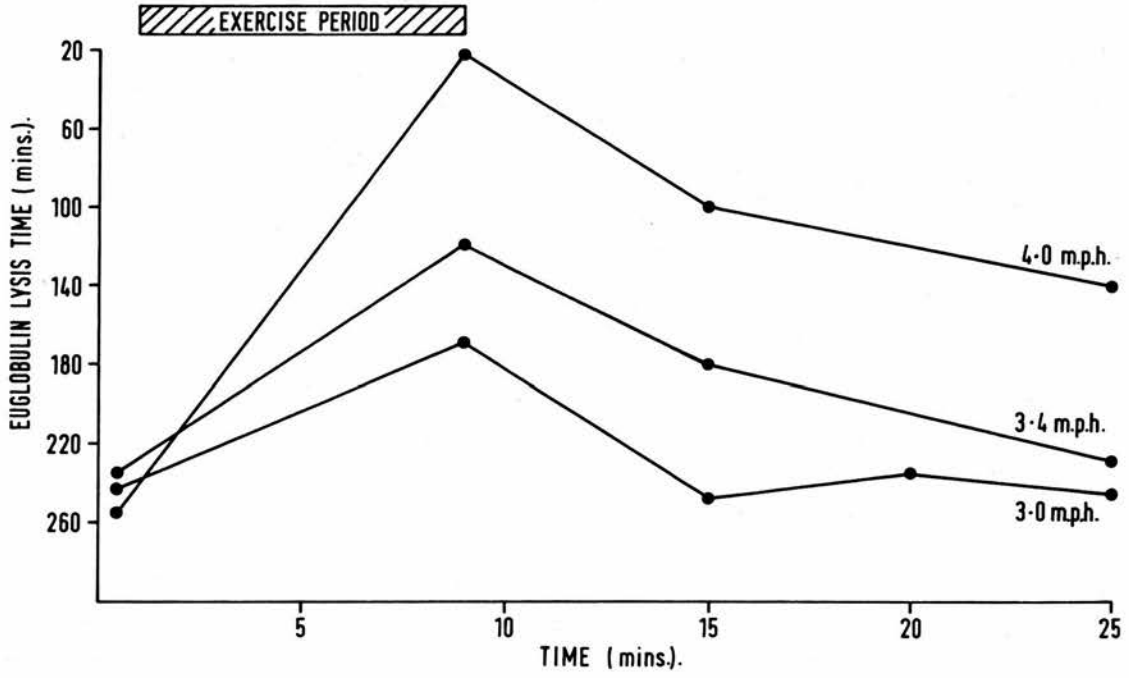

Fig.14. The euglobulin lysis times of a subject before and after walking at different speeds at a fixed elevation of 5°.

groups may prove to be an important observation. More detailed studies are indicated in which subjects are compared following exercises of comparable energy expenditures: such a project would prove to be a formidable task.

Cigarette smoking appeared to have no influence on the fibrinolytic response to exercise.

CHAPTER 8

RESULTS III

THE FIBRINOLYTIC RESPONSE TO EXHAUSTIVE EXERCISE

Six middle-aged male subjects, three known to be poor responders (10-20%) and three moderate responders (40-50%) to the standard exercise procedure, were studied before and after an exercise procedure designed to achieve exhaustion within an 8 minute period. This involved walking on the treadmill at an elevation of 10° for 3 minutes at 3.4 m.p.h. followed by 3 minutes at 3.7 m.p.h., then 1 minute at 4.0 m.p.h. and if possible a further minute at 4.2 m.p.h.

All subjects spontaneously terminated the exercise between the 50-60 second period of the final minute. The mean post-exercise pulse rate was 198/min. The results of the euglobulin lysis times before and after the exercise are shown in Table 8.

Euglobulin Lysis Time (min.)		%
Before	After	Increase
141	16	89
120	22	82
321	59	82
*765	413	46
*525	300	43
*730	340	53

TABLE 8. Fibrinolytic response to exhaustive exercise in 3 known moderate responders and 3 known *poor responders to the standard exercise procedure.

Comment

The purpose of this small series of experiments was to demonstrate that, even under extreme exhaustive conditions, it was still possible to differentiate between the good and bad responders. These observations confirm the finding of a plasminogen activator production defect in these poor responders and suggest that the standard exercise procedure could be used for further epidemiological studies.

CHAPTER 9

RESULTS IV

FIBRINOLYTIC RESPONSE TO MODERATE EXERCISE AND INTRAVENOUS ADRENALINE IN THE SAME SUBJECTS

Exercise has been shown to increase plasma adrenaline, and there appears to be some individual variation in the level of increased adrenaline following a standardised submaximal exercise procedure (Von Euler and Hellner 1952, Vendsalu 1960). Thus it is possible that the fibrinolytic response to exercise might be mediated by the stimulating effects of adrenaline and that the individual differences in fibrinolytic response are related to differences in adrenaline production. Information on this problem would be of importance from a physiological point of view, and might assist in a more satisfactory interpretation of the significance of the poor responders. Adrenaline also accelerates blood coagulation in vivo (Vosburgh and Richards 1903, Cannon and Gray 1914, Waldron 1951, Forwell and Ingram 1957). Thus, should the poor fibrinolytic responder be explained on the basis of poor adrenaline production, then it might be assumed that his coagulation response would also be minimal and therefore a coagulation fibrinolysis disequilibrium, with all its

proposed dangerous sequelae (Astrup 1956), would be unlikely.

The absence of facilities to measure blood catecholamines necessitated the use of an indirect method of studying this problem: a comparison between the fibrinolytic responses to exercise and a standard dose of intravenous adrenaline.

The subjects were healthy young male and female volunteers aged between 18 - 30 years. Twenty-five subjects were studied, 14 male (mean age: 23.4 years) and 11 female (mean age: 21.1 years). All subjects undertook the standard exercise procedure described in the previous chapters, and the intravenous adrenaline procedure described in Chapter 4, on more than one occasion. Most experiments were performed at weekly intervals, but for 3 subjects the interval between the last exercise procedure and the first adrenaline infusion was several months. Subject preparation was identical to previous exercise studies.

1. Pattern of fibrinolytic response to intravenous adrenaline

Fig. 15 demonstrates that the maximum fibrinolytic response, as assessed by the euglobulin lysis time, occurred 2 minutes after the termination of the 2 minute adrenaline infusion. On the basis of these results,

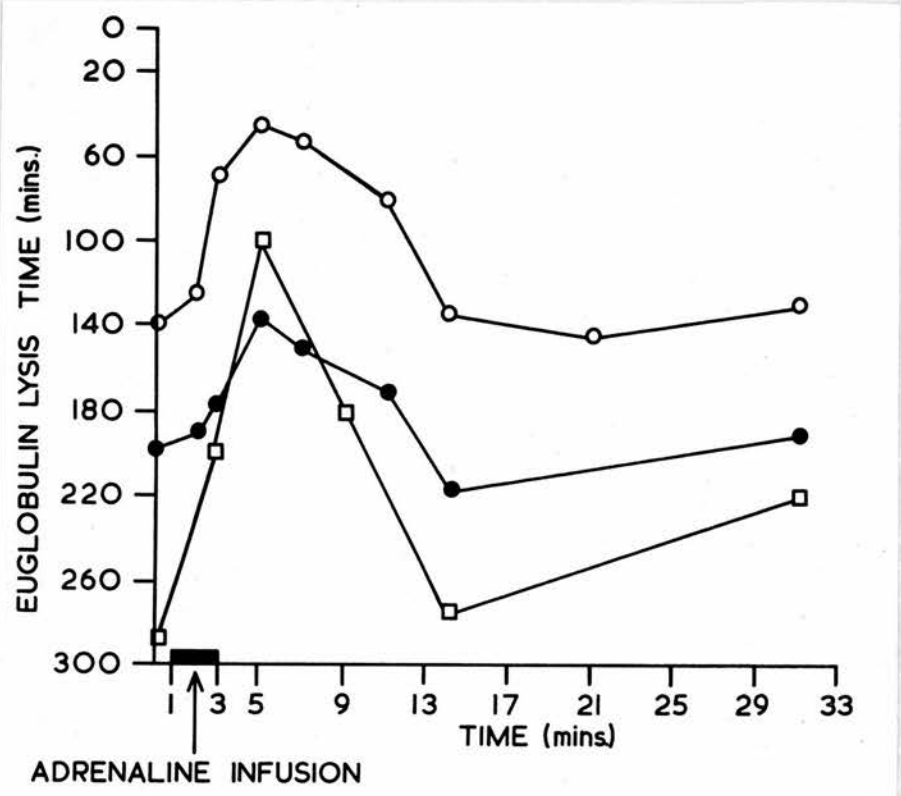

Fig.15 The euglobulin lysis time before, during and after adrenaline infusion in 3 subjects.

blood samples obtained for the study of the fibrinolytic response to adrenaline were withdrawn 1 minute before and 2 minutes after the termination of the adrenaline infusion.

2. Correlation of exercise and adrenaline stimulated fibrinolytic responses

Table 9 summarises the results of all experiments, showing the euglobulin lysis times before and after the exercise and adrenaline procedures. The percentage fibrinolytic responses were calculated as described previously.

The results show good individual reproducibility in response, both to exercise (correlation coefficient $r = 0.9929$ and $p < 0.001$) and to intravenous adrenaline (correlation coefficient $r = 0.9949$ and $p < 0.001$), and a significant degree of variation in response between individuals (analysis of variance for exercise $F = 48$ and intravenous adrenaline $F = 22$, both being significant at the 1% level).

Fig. 16 is a plot of the correlation between the percentage fibrinolytic response to exercise and intravenous adrenaline in all subjects. A highly significant correlation was demonstrated (correlation coefficient $r = 0.855$ and $p < 0.001$).

TABLE 9

THE FIBRINOLYTIC RESPONSE TO EXERCISE AND INTRAVENOUS ADRENALINE IN 25 YOUNG HEALTHY SUBJECTS

EXERCISE					ADRENALINE			
Subj. No.	Euglobulin Lysis Time (mins.)		% Increase	Mean % Increase	Euglobulin Lysis Time (mins.)		% Increase	Mean % Increase
	Before	After			Before	After		
1	140	71	49		165	68	61	
	660	332	50	49	104	48	54	58
	500	257	49					
2	620	440	29		210	113	46	
	84	60	29		257	141	45	46
	270	200	26	29				
	318	213	33					
	210	156	26					
3	115	70	39		107	62	41	
	146	96	34	37	115	68	41	41
4	550	440	20		565	510	9	
	211	172	19	24	655	505	22	16
	745	505	32					
5	143	60	58		250	90	65	
	150	65	57	58	157	57	64	65
	150	62	59					
6	105	63	40		125	71	43	
	95	59	38	40	56	32	43	43
	103	60	42					
7	120	95	21		270	185	32	
	159	116	27		160	92	42	37
	272	192	29					
	151	106	30	26				
	245	185	24					
	132	98	26					
8	112	52	54		91	32	65	
	223	104	53	54				65
	180	80	56					
9	72	65	10		97	57	41	
	108	86	20		80	56	30	35
	200	184	8	10				
	320	310	3					
10	189	99	48		150	55	63	
	270	137	49	49	113	48	59	61

TABLE 9 - continued

EXERCISE					ADRENALINE			
Subj. No.	Euglobulin Lysis Time (mins.)		% Increase	Mean % Increase	Euglobulin Lysis Time (mins.)		% Increase	Mean % Increase
	Before	After			Before	After		
11	65 84 94	51 56 64	22 33 32	29	86 83 73	46 45 40	46 45 47	46
12	116 108 110	87 82 85	25 24 23	24	105 98	60 58	42 41	42
13	174 135 100	89 77 60	49 43 40	44	88 140 96 215	46 70 50 103	48 50 47 50	49
14	100 115	50 55	50 52	51	92 134 115 140	37 46 40 45	59 65 65 60	62
*15	156 228 198	71 100 95	54 56 52	54	103 111 150	43 47 60	60 58 60	59
*16	112 113 99 97	70 75 62 61	38 34 37 37	39	99 100	54 55	45 45	45
*17	115 88 177 140	55 45 96 74	50 49 46 47	48	120 90	52 40	57 56	57
*18	222 135 204 192 193 143	97 71 90 95 97 70	56 47 56 51 50 51	52	120 122 105	55 57 51	54 53 51	53
*19	123 131 132 120	72 75 74 75	42 43 44 38	42	109 119 140	54 53 62	50 56 55	54
*20	132 135 145 142	62 65 71 62	53 52 51 49	51	143 104	56 59	58 54	56

*Female Subjects

TABLE 9 - continued

EXERCISE					ADRENALINE			
Subj. No.	Euglobulin Lysis Time (mins.) Before	After	% Increase	Mean % Increase	Euglobulin Lysis Time (mins.) Before	After	% Increase	Mean % Increase
*21	180	92	49	52	237	147	39	40
	306	142	54		290	150	36	
	250	117	53		151	81	46	
	148	71	52					
*22	595	380	36	39	139	57	59	57
	185	110	41		122	56	54	
	90	53	41					
*23	105	45	57	51	126	45	66	58
	110	55	50		117	57	51	
	100	50	50		95	42	56	
	105	55	48					
*24	150	76	49	47	126	48	62	57
	198	93	51		101	46	55	
	178	87	45		120	44	55	
	178	95	41					
*25	68	30	56	56	77	49	37	36
	80	33	59		54	37	32	
	82	32	56		54	33	39	
	89	39	54					

*Female Subjects

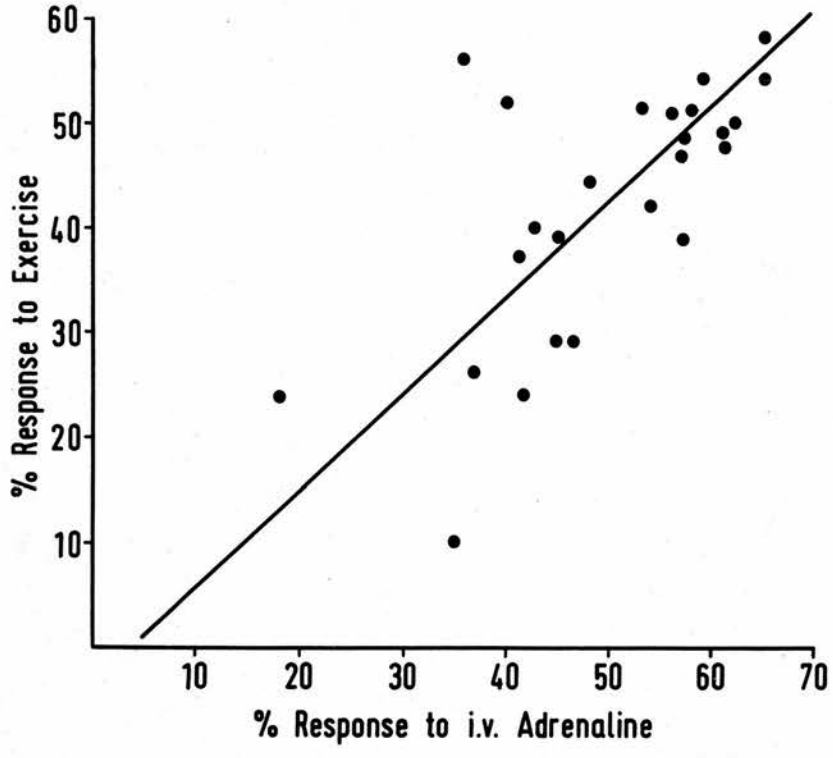

Fig.16. Correlation between the percent increase in euglobulin lysis time to moderate exercise and i.v. adrenaline.

$r = 0.855$, $p < 0.001$, $y = 0.92x - 3.4$

Comment

The demonstration of a good correlation between the fibrinolytic response to a standard exercise and intravenous adrenaline would seem to indicate, indirectly, that the variation in fibrinolytic response to exercise between individuals cannot be explained entirely on the basis of individual variability of adrenaline production to exercise. These findings are regarded as further proof of the existence of a small group of apparently healthy young persons whose mechanisms for increasing circulating plasminogen activator to stress appears to be defective. No data are at present available which can elucidate the mechanics of this defect. It is possible that it might arise due to an abnormality in the mechanisms governing the release of the plasminogen activator from the available stores in the vessel walls (Todd 1959, Warren 1963, Neri Serneri et al. 1965), or because these stores are consistently depleted.

CHAPTER 10

RESULTS V

THE EFFECT OF MENTAL STRESS ON FIBRINOLYTIC REACTIVITY TO EXERCISE

During the earlier part of this work it was assumed that the ability to generate plasminogen activator following exercise was, in part, genetically determined. Subsequent studies, reported in this chapter, would indicate that at least one environmental factor, in the form of mental stress, can influence this phenomenon.

Five male medical student volunteers were studied before, during and after a resit examination for the 2nd M.B., Ch.B. Five age and sex matched control volunteers were studied simultaneously. The control group were not sitting examinations, but enjoying their summer vacation.

The mean percentage fibrinolytic responses to the standard exercise procedure in the control subjects were 39, 47, 52, 50 and 36 respectively. There was good individual reproducibility throughout the period of study.

The results of the examination candidates are summarised in more detail in Table 10 and shown graphically in Fig. 17. Three of the 5 subjects, immediately prior to or during the examination period, failed to respond,

TABLE 10

THE FIBRINOLYTIC RESPONSE TO A MODERATE EXERCISE PROCEDURE BEFORE, DURING AND FOLLOWING AN EXAMINATION

Subject No.	Day of experiment in relation to onset of examination	Euglobulin Lysis Time (mins.)		% Increase in E.L.T.	Euglobulin on Unheated Fibrin Plates (area in $m.m.^2$)		Euglobulin Fibrinogen (mg./100 ml.)		Euglobulin Plasminogen (casein units/ml.)	
		Before	After		Before	After	Before	After	Before	After
S 1	-20	195	120	38	342	462				
	-12	105	58	44	419	623	148	161	3.25	3.40
	+ 1	135	80	40	310	441	156	160	3.14	3.27
	+ 4	115	65	43	500	650	134	139	3.42	3.49
	+21	77	45	41	506	650				
	+40	83	51	38	380	484				
	+60	148	92	39						
S 2	-21	430	297	31	245	320	262	284	4.18	4.36
	-14	620	440	29	190	270				
	- 7	568	508	11	156	169	200	218	3.98	4.21
	+ 2	405	415	0	255	260	226	242	4.01	4.23
	+ 6	84	60	28	470	500	224	238	4.13	4.30
	+21	270	194	28	272	361				
	+28	318	213	33	260	349	244	258	4.00	4.23
	+35	210	156	26	320	400				
	+56	414	290	30	250	315				
	+70	342	236	31						
S 3	-14	61	35	43	568	849	224	248	3.04	3.20
	- 7	75	42	44	420	613	244	260	2.98	3.16
	+ 4	82	84	0	550	550	200	216	2.99	3.15
	+ 7	120	94	21	300	400	214	230	3.09	3.28
	+18	64	47	27	410	480				
	+40	57	35	40	528	700	208	226	3.10	3.30
	+47	65	38	44	400	566				
	+63	81	45	44						
S 4	-21	105	60	43	441	572	114	128	2.61	2.80
	-10	131	78	41	361	484	108	112	2.76	2.94
	+ 1	77	47	39	400	529	120	144	2.88	2.99
	+ 4	76	50	35	462	570				
	+49	100	60	40	410	590				
S 5	-14	550	440	20	152	164	234	252	4.06	4.19
	- 4	560	570	0	170	162	216	230	4.11	4.29
	+ 7	211	172	18			226	244	4.10	4.26
	+21	326	238	27	206	294	230	250	4.11	4.30
	+33	745	505	32	152	180				
	+56	247	153	38	290	370				
	+70	492	325	34						

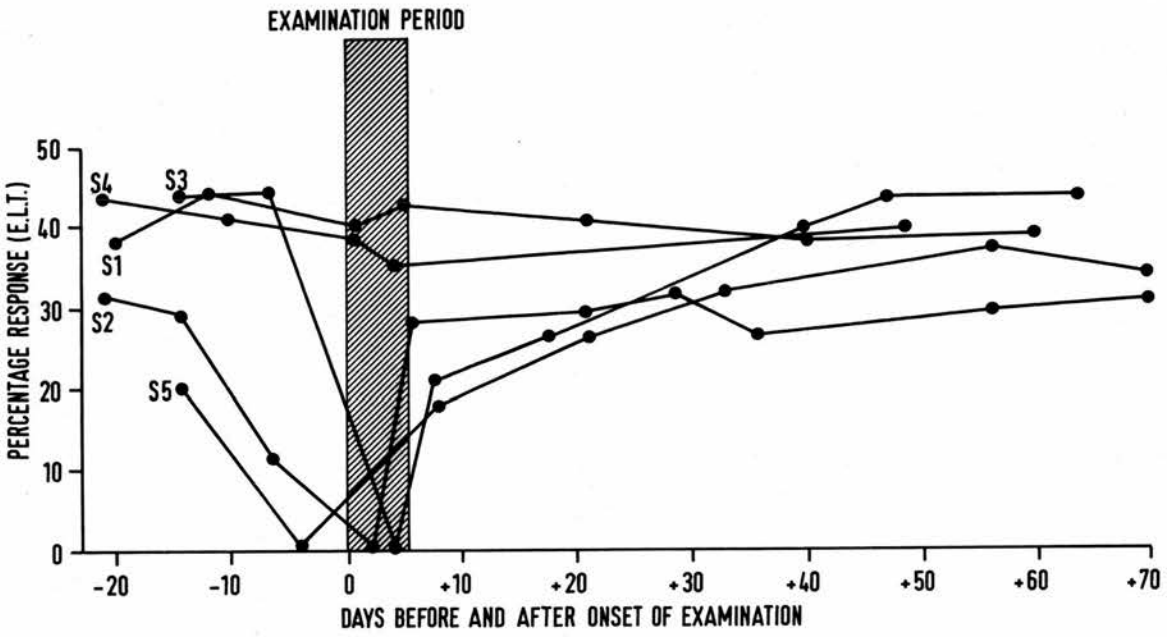

Fig.17. PERCENTAGE RESPONSE (E.L.T.) TO MODERATE EXERCISE IN 5 MALE SUBJECTS BEFORE, DURING AND AFTER AN EXAMINATION.

whereas the remaining 2 showed no such deterioration. This failure of fibrinolytic response could not be explained by a fall in plasminogen, rise in fibrinogen or the presence of a urokinase inhibitor in the post-exercise euglobulin solutions. The findings of the euglobulin solutions tested on unheated human fibrin plates would further support the view that a production defect of plasminogen activator had occurred.

Comment

The results of this small study would indicate that prolonged mental stress may produce a deleterious influence on a normally good fibrinolytic reactivity and that this effect may persist to a lesser degree for some weeks beyond the end of the crisis. Of no less interest was the observation that 2 of the 5 subjects showed no significant change in their fibrinolytic reactivity throughout the period of the study. The author was unable to convince himself that these 2 subjects were less anxious than their 3 colleagues. There was no correlation between the presence or absence of the deleterious effect on the fibrinolytic reactivity and the results of the degree examination.

The mechanism of this effect of prolonged mental stress on fibrinolytic reactivity to exercise remains obscure. Acute anxiety stimulates fibrinolysis

(Macfarlane and Biggs 1946, Truelove 1951, Schneider and Zangari 1951, Ogston et al. 1962, Ogston 1964, Patsch 1963), and it is possible that prolonged mental stress may effect a partial exhaustion of the production mechanisms which are responsible for the post-exercise increase. Such exhaustion has been proposed following prolonged exercise (Ogston and Fullerton 1961) and severe trauma (Bergentz and Nilsson 1961, Innes and Sevitt 1964).

The absence of a significant fall in the resting level of plasminogen activator, as measured by the euglobulin lysis time, coincident with this deleterious effect on the fibrinolytic reactivity to exercise, might be regarded as failing to substantiate the hypothesis of partial exhaustion of the activator input system. It is possible, however, that either the liver, which controls the removal of circulating plasminogen activator (Fletcher et al. 1964, Janusko et al. 1966), can compensate by reducing activator clearance, or the production of plasminogen activator which governs the resting level of fibrinolytic activity may be largely contributed to by sources or control mechanisms not directly related to the exercise response phenomenon. This latter concept has support from the work of Amery et al. (1965) in their studies using a niacin compound as the fibrinolytic stimulant.

CHAPTER 11

RESULTS VI

THE FIBRINOLYTIC RESPONSE TO PROLONGED EXERCISE

The standard exercise and adrenaline procedures, described in the previous chapters, were of relatively short duration. It might be argued that poor responders to this limited challenge could compensate when a prolonged stimulus was applied.

This problem was studied in 11 volunteers, whose fibrinolytic response to the standard exercise procedure had been determined previously, by exercising them for 3 hours on a level treadmill at 4.00 m.p.h. following a 30 minute rest period. At hourly intervals the treadmill was stopped for 30 seconds, during which time a sample of blood was obtained from a cubital vein. Fluid in the form of orange juice was provided ad lib.

The results are summarised in Tables 11 and 12 and graphically in Fig. 18. The fibrinolytic activity, as measured by the euglobulin lysis time and fibrin plate method, increased during the exercise procedure and plateaued at 2 hours in all but one subject. One subject (I.F.) was exercised for a further 2 hours and during this

After P. 90.

TABLE 11

THE PLASMINOGEN ACTIVATOR RESPONSE TO A PROLONGED TREADMILL EXERCISE PROCEDURE

Subject	Mean % Response to Standard Exercise	BEFORE			1 Hr.			2 Hr.			3 Hr.		
		Mean E.L.T. (mins)	Area on Fibrin Plates (mm^2)		Mean E.L.T. (mins)	Area on Fibrin Plates (mm^2)		Mean E.L.T. (mins)	Area on Fibrin Plates (mm^2)		Mean E.L.T. (mins)	Area on Fibrin Plates (mm^2)	
			Euglobulin	Plasma		Euglobulin	Plasma		Euglobulin	Plasma		Euglobulin	Plasma
G.McK.	60	780	81	0	81	342	81	40	506	100	41	510	100
A.McG.	58	397	225	42	123	400	81	65	484	144	52	576	169
P.B.	54	123	340	--	52	472	---	42	524	---	42	529	---
D.F.	52	115	462	0	62	525	100	41	629	225	45	650	225
D.M.	50	520	196	0	120	450	---	48	750	278	47	750	225
J.D.	50	77	---	--	50	---	---	45	---	---	40	---	---
I.F.	40	132	400	25	67	515	196	50	552	200	41	576	210
D.J.	39	340	324	0	75	462	49	42	612	100	42	600	100
I.W.	38	222	340	0	99	550	121	79	529	156	80	527	156
W.G.	10	234	324	49	85	484	110	56	589	121	56	600	132
M.A.	10	808	81	0	476	225	0	256	300	64	120	289	81

TABLE 12

CHANGES IN EUGLOBULIN FIBRINOGEN AND PLASMINOGEN AND PLASMA UROKINASE INHIBITOR BEFORE AND AFTER A PROLONGED TREADMILL EXERCISE PROCEDURE

Subject	Euglobulin Fibrinogen (mg.%)		Euglobulin Plasminogen (c.u./ml.)		Fibrin Plate Inhibitor Assay (Area in mm^2)			
					Before		3 Hrs.	
	Before	3 Hrs.	Before	3 Hrs.	$\frac{1}{50}$	$\frac{1}{100}$	$\frac{1}{50}$	$\frac{1}{100}$
D.M.	250	252	4.55	4.51	100	132	81	110
G.McK.	194	187	3.01	3.04	144	225	144	225
I.W.	320	301	3.25	3.31	100	175	100	195
I.F.	182	191	2.10	2.40	---	---	---	---
D.F.	272	282	3.27	3.10	---	---	---	---

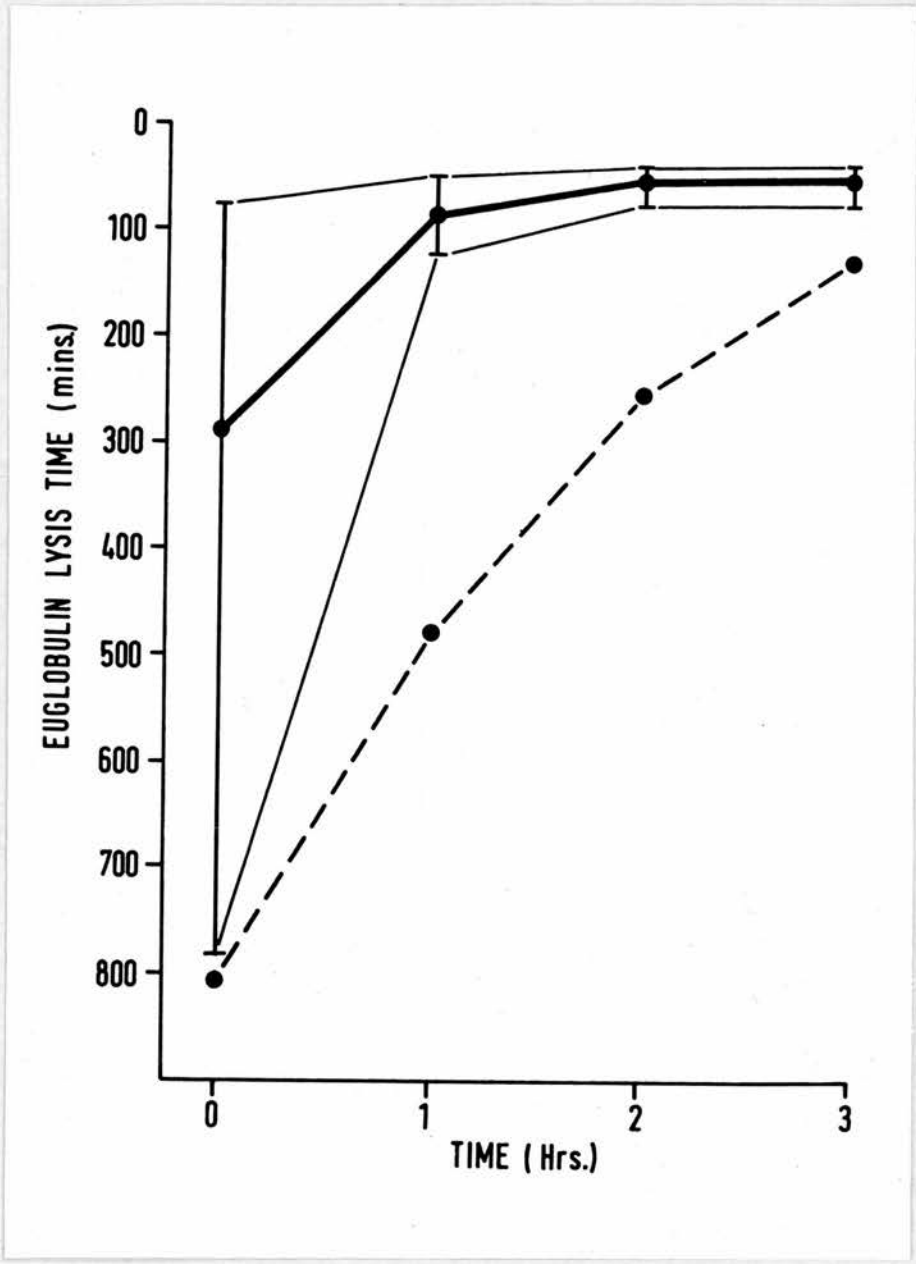

Fig. 18. The euglobulin lysis time before and during a period of prolonged exercise. The continuous line represents the mean, with the range, of 10 subjects. The interrupted line represents the response of subject M.A. (see text).

time no further increase or subsequent decrease in the level of fibrinolysis occurred. There was no significant difference in euglobulin plasminogen, fibrinogen or plasma urokinase inhibitor levels before and after the exercise procedure in those subjects studied (Table 12).

Comment

These results confirm the finding of Ogston and Fullerton (1961) that a steady state of fibrinolysis following prolonged exercise is usually reached at approximately the 2 hour mark. This observation was not, however, applicable to one subject (M.A.) who, assuming he could reach the level of the other subjects, had not plateaued even at the 3 hour mark (Fig. 18). This finding could not be explained by the low level at which he started prior to the exercise, for comparison with another subject (G.McK.), whose pre-exercise level was similar, shows the marked difference between these two subjects (Fig. 19).

It is of some interest to note that subject M.A. had also been shown to be a poor responder to both the standard and the exhaustive exercise procedures. However, another subject (W.G.), while known to be a poor responder to short duration procedures, displayed a response similar to the normal group in the prolonged exercise situation. This would indicate that there are two types of poor responder: those who show an abnormal response to short

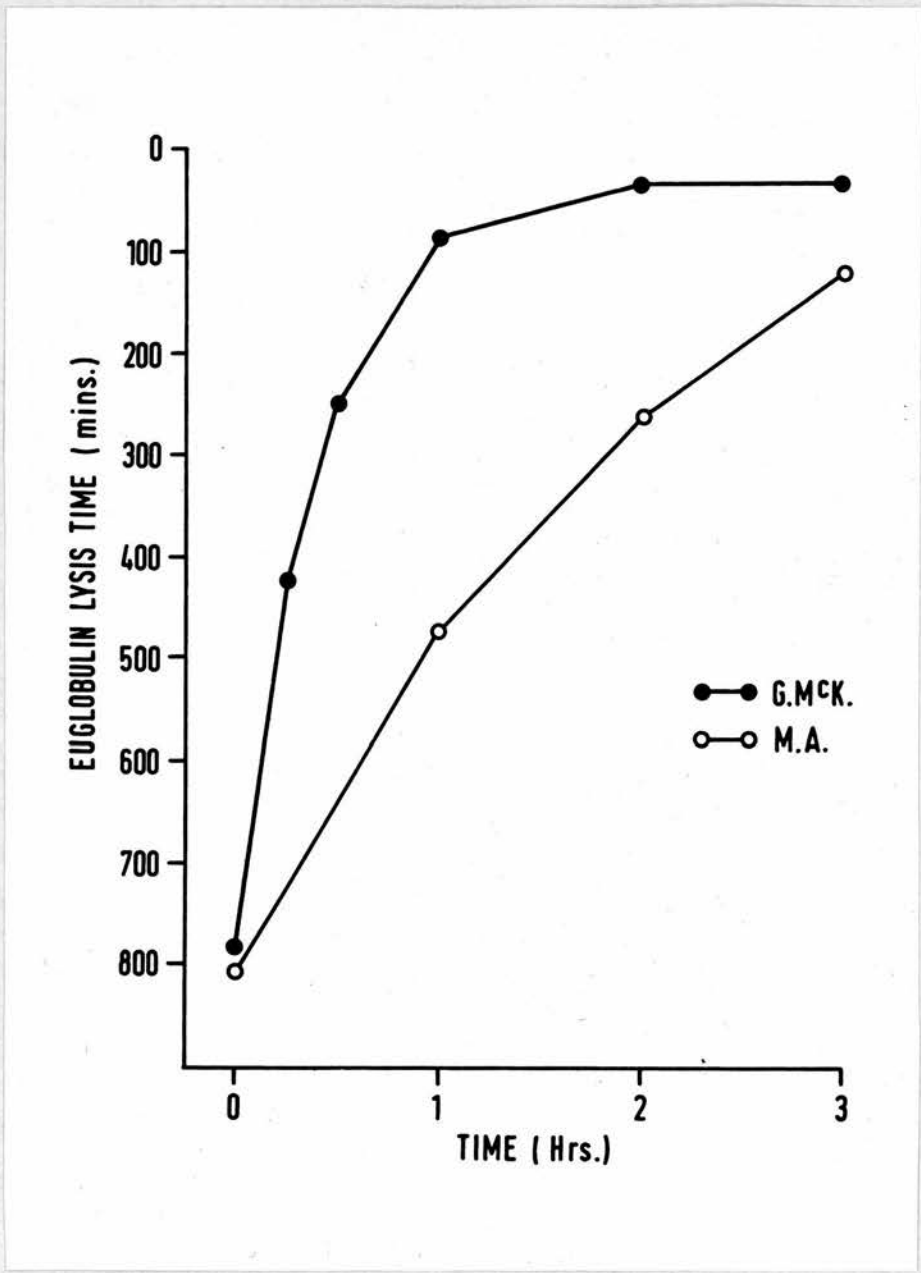

Fig. 19. A comparison of the fibrinolytic response to the prolonged exercise procedure between two subjects (G.McK. and M.A.) whose pre-exercise euglobulin lysis times were similar.

duration stress only, and those whose response is defective to both long and short term stimulation.

The finding that, despite a wide range in the resting euglobulin lysis times (77-780 min.) in the 10 remaining subjects, the final time plateaued between 40 - 80 minutes was unexpected but of considerable interest. It is suggested that this observation would indicate that a comparison of euglobulin lysis times between normal individuals, provided the assay techniques are satisfactory, is valid. It is also proposed that this finding could be interpreted as demonstrating that the difference in resting levels of plasminogen activator between individuals may represent normal differences in the homeostatic mechanisms controlling the complex multifactorial in vivo thrombolysis. Thus the level of circulating activator in the resting state would not be necessarily a mirror of the level of active thrombolysis for the individual susceptibility of deposited fibrin to lysis could be an important modulating factor. In situations of stress, however, when rapid dynamic changes in fibrinolysis may be required, the ability of the individual to augment the level of activator could be the most important single factor.

Further progress in this aspect of fibrinolysis is unlikely without new techniques which make it possible to assess the level of overall in vivo thrombolysis. The recent introduction of a sensitive immunological assay of fibrin/fibrinogen degradation products (Merskey et al.

1966) may prove to be an invaluable tool in future studies of this problem.

CHAPTER 12

CONCLUSIONS

The significance of the fibrinolytic reactivity to exercise and adrenaline remains, as yet, unknown. It is postulated that it may represent, in part, the ability of the individual to generate circulating plasminogen activator to stress. Exercise and adrenaline have also been shown to increase the coagulability of the blood (Vosburgh and Richards 1903, Cannon and Gray 1914, Vuori 1950, Waldron 1951, Forwell and Ingram 1957, Bond et al. 1961, Iatridis and Ferguson 1963, Burt et al. 1964, Von Kaulla and Von Kaulla 1964). Thus, on the basis of a dynamic equilibrium between coagulation and fibrinolysis, an increase in fibrinolysis would be anticipated. Iatridis and Ferguson (1963), however, observed that 11 of their 59 subjects failed to increase their circulating plasminogen activator following strenuous exercise despite an apparent increase in coagulability. Burt et al. (1964), in a study of coagulation and fibrinolysis following strenuous exercise, concluded that the mean changes were in favour of fibrinolysis. Examination of their results in more detail, however, shows that two of their 44 subjects gave a poor fibrinolytic response, whereas the coagulation changes in these subjects were similar to those in some of the subjects giving a marked fibrinolytic response.

Hypercoagulability and increased fibrinolysis have also been demonstrated during acute anxiety (Ogston et al. 1962, Patsch 1963). It is suggested that the possibility now arises that persons with a poor fibrinolytic reactivity may be subject to transitory episodes of coagulation-fibrinolysis disequilibrium during their day-to-day living. The disequilibrium might arise from either an exaggerated increase in coagulation associated with a normal increase in fibrinolysis or vice versa. In the light of Astrup's hypothesis these persons may be at risk to atherosclerosis and/or thrombosis.

Evidence supporting this view is fragmentary. Cirrhotic patients have a low incidence of myocardial infarction (Grant et al. 1959, Howell and Manion 1960), and it has been shown that they may possess exaggerated fibrinolytic reactivity (Weiner 1963, Fletcher et al. 1964) as well as a general tendency to hypocoagulation (Ollendorff et al. 1966). Von Kaulla and Von Kaulla (1964) have provided suggestive evidence that patients with a proven history of myocardial infarction can show an exaggerated hypercoagulability after exercise.

There is reason to believe that atherosclerosis is prevalent in young males (Thomas 1957, Holman et al. 1958) and that women, during the reproductive period of their lives, are relatively immune (Thomas 1957, Strong and McGill 1962). The finding of a small group of apparently normal young men with poor fibrinolytic reactivity to

moderate exercise, and of women in the same age-group with a greater fibrinolytic reactivity, may prove to be of some interest. Sufficient data are not available to compare the effects of age on this phenomenon.

The role of mental stress in atherogenesis and thrombosis remains controversial. Three mechanisms have been postulated: intimal trauma by repeated 'whiplash' effects on unsupported parts of the coronary arterial trunks (Hickman et al. 1958, Texon 1957, Enos et al. 1962), the production of a hyperlipaemia (Mysamnikov 1958, Cardon and Gordon 1959) and the acceleration of coagulation (Schneider and Zangari 1951, Dreyfuss 1956, Friedman a et al. 1958). Previous studies on fibrinolysis during university examinations have failed to show consistent changes in the absolute resting levels of plasminogen activator (Truelove 1951, Truelove 1953, Sherry et al. 1959, Sawyer et al. 1960). The results of the present investigation have confirmed these findings but have revealed a striking abnormality in the fibrinolytic response to exercise during the examination period. In the light of the hypothesis previously discussed, on the biological significance of the fibrinolytic reactivity to exercise, these results may prove to have important consequences to further studies on the relationship of prolonged mental stress, fibrinolysis and occlusive vascular disease.

Although the scope of this study was necessarily limited, sufficient information has been made available

which indicates that the capacity of an individual to generate circulating plasminogen activator to various physiological stimuli may have important bearings on any further understanding of the physiological control mechanisms of the fibrinolytic enzyme system. It is not related to absolute resting levels of fibrinolysis and may prove to play a significant role in the proposed relationships between fibrinolysis and occlusive vascular disease, the metastasis of malignant tumours and recovery from severe shock.

APPENDIX

APPENDIX

ANTHROPOMORPHIC DATA OF SUBJECTS

Subject Initials	Subject Number*	Sex	Age	Weight (lbs).	Height (inches)
G.McK.	M 1	M	23	164	72
J.D.	M 2	M	27	163	70
I.McK.	M 3	M	23	170	73
D.J.	M 4	M	23	154	71
P.S.	M 5	M	24	158	73
I.F.	M 6	M	24	141	71
J.B.	M 7	M	22	175	71
M.P.	M 8	M	29	175	71
W.G.	M 9	M	30	174	71
I.W.	M 10	M	23	140	70
I.N.	M 11	M	22	163	73
R.W.	M 12	M	24	140	69
D.M.	M 13	M	23	149	73
A.P.	M 14	M	25	161	71
D.F.	M 15	M	19	136	69
I.A.	M 16	M	22	142	67
H.M.	M 17	M	24	168	72
G.A.	M 18	M	26	175	68
G.W.	M 19	M	30	140	72
A.M.	M 20	M	26	168	71
A.W.	M 21	M	23	140	69
P.K.	M 22	M	23	168	72
J.C.	M 23	M	30	171	70
S.D.	M 24	M	22	160	72
M.G.	M 25	M	22	150	69
A.R.	F 1	F	19	110	62
M.F.	F 2	F	21	136	64
J.B.	F 3	F	25	133	68
E.C.	F 4	F	30	150	65
J.T.	F 5	F	30	126	70
F.Sp.	F 6	F	20	144	67
R.W.	F 7	F	20	147	69
H.S.	F 8	F	19	143	62
R.L.	F 9	F	19	112	66
K.A.	F 10	F	19	129	63
S.E.	F 11	F	21	128	66
T.G.	F 12	F	20	141	66

Subject Initials	Subject Number*	Sex	Age	Weight (lbs)	Height (inches)
A.B.	F 13	F	20	154	69
A.H.	F 14	F	20	126	64
P.B.	F 15	F	23	136	67
I.B.	F 16	F	22	147	62
L.W.	F 17	F	19	140	67
M.L.	F 18	F	19	109	62
F.S.	F 19	F	19	133	62
M.K.	F 20	F	19	112	62
C.W.	F 21	F	23	112	63
I.S.	F 22	F	23	140	67
M.H.	F 23	F	22	114	63
M.Law.	F 24	F	24	112	65
E.A.	F 25	F	25	140	65
M.A.	1	M	46	203	69
J.D.	2	M	50	190	72
B.C.	3	M	42	187	70
R.C.	4	M	58	146	68
T.D.	5	M	50	172	66
A.E.	6	M	48	174	67
B.L.	7	M	48	136	67
T.W.	8	M	54	156	67
S.R.	9	M	55	196	71
P.B.	10	M	47	163	70
W.T.	11	M	50	166	66
T.L.	12	M	46	161	68
D.G.	13	M	50	177	68
C.G.	14	M	45	208	72
J.B.	15	M	52	196	73
J.L.	16	M	49	172	70
C.D.	17	M	55	169	64
R.A.	18	M	46	182	73
T.S.	19	M	57	187	71
G.G.	20	M	54	135	64
P.C.	21	M	53	175	67
J.P.	22	M	44	185	71
W.G.	23	M	54	161	67
J.K.	24	M	50	173	70
W.O'N.	25	M	46	161	70
W.H.	1	F	54	119	62
J.M.	2	F	55	127	61
H.W.	3	F	49	171	65
P.R.	4	F	54	116	62
C.P.	5	F	50	145	63
C.M.	6	F	53	133	62
M.D.	7	F	46	167	66

Subject Initials	Subject Number*	Sex	Age	Weight (lbs)	Height (inches)
A.A.	8	F	46	185	66
I.McL.	9	F	51	140	65
E.T.	10	F	51	128	62
E.R.	11	F	42	121	61
A.W.	12	F	45	158	65
L.C.	13	F	47	116	63
J.W.	14	F	45	143	64
E.B.	15	F	44	137	63
M.B.	16	F	47	154	68
M.H.	17	F	56	145	70
A.B.	18	F	57	140	61
Mgt.W.	19	F	51	112	59
J.S.	20	F	53	119	62
J.McM.	21	F	45	134	63
A.P.	22	F	47	158	63
H.Y.	23	F	45	154	66
J.A.	24	F	57	168	60
M.G.	25	F	50	112	63

*Subject number refers to those of the exercise studies

STATISTICAL METHODS

1. **Student t-test**

$$V = \frac{1}{n_1+n_2-2} \left(\Sigma x_1^2 - \frac{(\Sigma x_1)^2}{n_1} + \Sigma x_2^2 - \frac{(\Sigma x_2)^2}{n_2} \right)$$

$$S.D. = \sqrt{V}$$

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S.D. \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

V = Variance.

S.D. = Standard Deviation.

$\bar{x}_1 - \bar{x}_2$ = Difference between the mean values of the groups under examination.

Σx_1^2, Σx_2^2 = Sum of the squared observations in groups 1 (x_1) and 2 (x_2).

$(\Sigma x_1)^2$, $(\Sigma x_2)^2$ = Sum of the observations of groups 1 (x_1) and 2 (x_2) all squared.

n_1, n_2 = Total observations in groups (n_1) and 2 (n_2).

p (probability) was obtained by observing the relationship between the total degrees of freedom minus one and the value of t in the students t - distribution tables*. Values of p equal to or less than 0.05 were regarded as significant.

2. Analysis of Variance

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares
Between Varieties	$\Sigma T_i^2/n_i - C$	K - 1	M
Residual Varieties	By Substraction	N - k	S^2
TOTAL	$\Sigma x_{ji}^2 - C$	N - 1	-

N = total number of observations.

G = (grand total) = Σx_{ji}.

C = (correction factor) = G^2/N.

$$M = \frac{\Sigma T_i^2/n_i - C}{k - 1}$$

$$S^2 = \frac{(\Sigma x_{ij}^2 - C) - (ET_i^2/n_i - C)}{N - k}$$

k = number of subjects.

T_i = total of each subject.

n_i = number of observations for each subject.

Significance levels were obtained by using the F - distribution tables* where the degrees of freedom for the numerator $f_1 = k - 1$ and the denominator $f_2 = N - k$.

3. Correlation Coefficient

$$r = \frac{\Sigma(x - \bar{x})(y - \bar{y})}{\sqrt{\Sigma(x - \bar{x})^2 \, \Sigma(y - \bar{y})^2}}$$

Where r = correlation coefficient.

x and y = each observation in groups x and y.

$\bar{x}$ and $\bar{y}$ = mean of observations in groups x and y.

The probability (p) of x and y being correlated was obtained by comparing the value of (r) with the degrees of freedom minus one in The Correlation Coefficient Tables*.

4. Fitting a Regression Line

The time regression line was taken as

$$y = a + bx$$

a and b were obtained as follows:-

$$b = \frac{\Sigma(x - \bar{x})(y - \bar{y})}{\Sigma(x - \bar{x})^2}$$

$$\text{and } a = \bar{y} - b\bar{x}$$

These results were inserted into y = a + bx and the regression line drawn for various arbitrary values of x.

*The statistical tables used were an abridged form from Statistical Tables for Biological, Agricultural and Medical Research, published by Oliver and Boyd Ltd., Edinburgh.

CONSTITUENTS OF REAGENTS

A. REAGENTS FOR FIBRINOGEN ESTIMATION

(1) Solution of -Amino-Caproic Acid (E.A.C.A.) in saline. (1 litre).

710 mgm. E.A.C.A. were dissolved in 1 litre of 0.9% saline.

(2) Solution of Thrombin 100 units/ml. in saline. (100 ml.).

The contents of 2 vials of Parke Davis thrombin were dissolved in a small quantity of 0.9% saline. The solution was transferred quantitatively to a 100 ml. volumetric flask, ensuring that the vials were thoroughly rinsed with 0.9% saline and made up to 100 ml. with 0.9% saline. 1.5 ml. volumes were dispensed into 5 ml. plastic vials and stored at -20° C.

(3) Solution of Calcium Chloride 2.5% in saline. (500 ml.).

12.5 gm. anhydrous calcium chloride were dissolved in 500 ml. 0.9% saline.

(4) Solution of Sodium Hydroxide 10%. (500 ml.).

50 gm. sodium hydroxide were dissolved in 500 ml. distilled water.

(5) Solution of Trichloroacetic acid 5%. (500 ml.).

25 gm. trichloroacetic acid were dissolved in 500 ml. distilled water.

(6) Solution of Sodium Hydroxide 0.5N. (500 ml.).

10.0 gm. sodium hydroxide were dissolved in 500 ml. distilled water and standardised against N hydrochloric acid solution.

B. REAGENTS FOR PLASMINOGEN ESTIMATION

(1) $^{N}/_{6}$ Solution of Hydrochloric Acid. (500 ml.).

7.5 ml. of concentrated hydrochloric acid were added to 500 ml. of distilled water and standardised against N NaOH using Bromothymol blue as indicator.

(2) $^{N}/_{6}$ Solution of Sodium Hydroxide. (500 ml.).

3.33 gm. of sodium hydroxide were dissolved in 500 ml.

distilled water and standardised against $^{N}/6$ HCl using Bromothymol blue as indicator.

(3) 0.1M Phosphate Buffer pH 7.6

(i) 13.6 gm. potassium dihydrogen phosphate were dissolved in 1 litre of distilled water (Solution A).

(ii) 14.2 gm. disodium hydrogen phosphate were dissolved in 1 litre of distilled water (Solution B).

1 litre 0.1M phosphate buffer pH 7.6 was prepared by mixing 870 ml. of Solution A and 130 ml. of Solution B.

(4) Streptokinase

The contents of 1 vial of Lederle Varidase Streptokinase-Streptodornase were dissolved in 10 ml. 0.1M phosphate buffer pH 7.6.

(5) Casein

(i) 65 gm. of Casein were boiled in 1 litre of a 50:50 mixture of 0.9% saline and 0.1M phosphate buffer pH 7.6 for 20 minutes. During the boiling the solution was stirred vigorously.

(ii) The casein solution was then dialysed against

running tap water overnight.

(iii) The casein solution was then dialysed against 0.1M phosphate buffer for 5-6 days. The buffer was changed twice daily.

(iv) After dialysis the casein solution was centrifuged to remove any undissolved material. A protein estimation was performed on the clear supernatant and final dilution to 5 gm.% with the 50:50 mixture of 0.9% saline and 0.1M phosphate buffer was made. The casein was then dispensed in 6.5 ml. quantities and stored frozen at -20° C. in plastic tubes.

C. GENERAL REAGENTS

(1) Tris Buffer pH 7.8. (4 litres).

73 gm. tris hydroxymethyl amino methane (Koch Light Laboratories) were dissolved in 2 litres of distilled water and the pH adjusted to 7.8 with N HCl. The volume was then made up to 4 litres with distilled water and the pH checked.

(2) Barbiturate Buffer pH 7.4.

(a) Stock buffer (Veronal acetate base)

9.714 gm. sodium acetate trihydrate and 14.714 gm. sodium diethyl barbiturate were dissolved in 500 ml. of distilled water and stored at +4° C.

(b) Working buffer (Veronal acetate buffer pH 7.4)

25 ml. of the stock veronal acetate buffer were mixed with 25 ml. 0.1 N HCl and made up to 500 ml. with 0.9% saline. The pH was adjusted to 7.4 with either 0.1 N HCl or 0.1 N NaOH. This working buffer was stored at +4° C.

(3) Thrombin Solution for E.L.T. (Solution of 5 units/ml. in Veronal Acetate Working Buffer: 2 litres).

The contents of 2 vials of Parke Davis thrombin (5000 N.I.H. units per vial) were dissolved in 2 litres of working veronal acetate buffer. This thrombin solution was stored in plastic tubes at -20° C. in 4 ml. aliquots.

(4) Thrombin Solution for Fibrin Plates (50 units/ml. in tris buffer, 200 ml.).

The contents of 2 vials of Parke Davis thrombin (5000 N.I.H. units per vial) were dissolved in 200 ml. tris buffer pH 7.8, and stored in 2 ml. aliquots at -20° C. in plastic vials.

(5) Concentrated Human Fibrinogen for Fibrin Plates

7.5 gm. human fibrinogen (Kabi) were dissolved in 500 ml. tris buffer pH 7.8, and stirred gently at 37° C. in a water-bath.

The solution was dispensed in 4.2 ml. and 1.1 ml. aliquots and stored in plastic tubes at -20° C. until required.

(6) Urokinase Control Solution for Fibrin Plates 3 units/ml. (800 ml.).

The contents of one vial of Urokinase (2400 Ploug units) were dissolved in 800 ml. tris buffer, and dispensed in 1 ml. aliquots in 5 ml. plastic tubes and stored at -20° C. until required.

CLEANING AND SILICONING OF GLASSWARE

1. CLEANING OF GLASSWARE

(a) All glassware was immersed for at least 24 hours in a strong detergent solution of Pyroneg (Diversey (U.K.) Ltd.).

(b) Boiled for 30 minutes in a strong solution of Pyroneg.

(c) Rinsed free of Pyroneg in running tap water for 5 hours.

(d) Immersed in chromic acid cleaning fluid for at least 24 hours.

(e) Rinsed free of chromic acid in running tap water for 12 hours.

(f) Rinsed in 3 changes of distilled water.

(g) Dried in a hot air oven overnight.

2. SILICONISATION OF GLASSWARE

(a) The glassware was totally immersed in a 3% solution of I.C.I. M550 silicone in trichlorethylene for 2 hours.

(b) Dried in a hot air oven overnight.

(c) Rinsed thoroughly in distilled water (6 changes).

(d) Dried in a hot air oven overnight.

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