THE FORMATION AND FATE OF THROMBI WITH PARTICULAR EMPHASIS ON THE ROLE OF THE PLATELET

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"THE FORMATION AND FATE OF THROMBI"

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Neville G. Ardlie, M.B., B.S., M.D.

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I wish to declare that this Thesis is entirely of my own composition. It has not been submitted to obtain any other degree or diploma from another University.

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January, 1967.

PART I

HISTORICAL SURVEY

PART I - HISTORICAL SURVEY

Introduction

According to Robb-Smith (1955) the word "thrombosis" was coined by the Greek physician Claudius Galen, but not used in its modern sense until the nineteenth century. Prior to this, it was probably employed in much the same manner as the Anglo-Saxon word "clott". However, after a significant series of experiments in the five years 1882 to 1887, which clearly established the important role of the platelet in the development of a solid mass of blood in an injured vessel, the term thrombosis began to take on a more precise meaning, while the words clotting and coagulation came to be restricted to the solidification of blood under static conditions.

The word clot is now used to describe the solid material which is formed when blood solidifies under static conditions, in the body after death, or when shed from the body. This solid material is soft, shiny and gelatinous in appearance, and is dark red in colour and homogeneous, unless sedimentation has occurred before clotting is complete, when cell-rich and plasma-rich zones are formed. Microscopy shows a homogeneous mass of red cells enmeshed in a fibrin network. Discrete white cells and platelets are present in the proportions in which they normally occur in the blood and are therefore inconspicuous.

On the other hand a thrombus is "a solid mass or plug formed in the living heart or vessels from constituents of the blood". By employing this definition Welch (1889), in a monumental review of the subject,

wished to indicate the important role of blood cells, and in particular platelets, in thrombosis. Microscopically a thrombus is not homogeneous. It consists of masses of platelet aggregates encrusted with white cells and fibrin, and between these aggregates, red cells enmeshed in fibrin. Grossly thrombi are firmer than clots, and are dry, friable and mottled in appearance, pale and dark red areas being visible.

Many thrombi, especially venous thrombi, consist of two different parts. The first part or head is formed in flowing blood by the aggregation of platelets and has a pale appearance. This either reduces or completely stops the blood flow, thereby facilitating the development of the second part or tail of the thrombus. This latter is red in colour and structurally is a blood clot.

Thus, although synonymous from an etymological point of view, we have come to distinguish between a clot and a thrombus. Regrettably this utopian view still lacks many adherents and even today there is a considerable intermingling of these two terms. The distinction has been and should be made for two reasons. Firstly there is the morphological difference. The importance of determining at necropsy whether a solidified mass of blood formed prior to, or after death is vital. For this reason alone a distinction is justified. In this circumstance it is obviously desirable to employ different terms for two solid objects whose clinical significance is so extremely different. Secondly, it has been proposed that the mode of formation is different. Subsequently evidence will be presented in this review that a thrombus might arise

in two different ways, depending on the presence or absence of vascular injury. The initial stages of thrombosis in an injured vessel appear to be independent of the coagulation mechanism. Alternatively, in the absence of preceding vascular injury thrombosis may represent a process of intravascular coagulation.

In 1846 Virchow outlined the principal factors influencing thrombosis. These were:

(1) the vessel wall

(2) the composition of the blood

and (3) the blood flow.

Although there have been considerable recent advances in our understanding of the mechanisms of thrombosis, this classic triad still remains valid today, and thrombogenesis can best be discussed under these three headings. Some overlap in discussion must result, but nevertheless this division is convenient.

(1) The vessel wall and thrombosis

Observations in the nineteenth century

The sequence of events leading to the formation of a thrombus were established in the second half of the nineteenth century largely by observations of the effects of injury to vessels in living animals. Wharton Jones in 1851 appears to have been the first to observe the earliest stages of thrombosis. During experiments on the inflammatory reaction in the web of the frog's foot he noticed the accumulation of cells at a site of vascular injury. He claimed that these cells were held together by fibrin. He also observed embolism of clumps of these

cells. Mantegazza (1869) and Zahn (1875) both observed the early phases of thrombosis in the mesentery of the frog, and like Wharton Jones regarded the first cells appearing at sites of vascular injury to be leucocytes. They may not have appreciated the observations of Donne (1842) who had already published a description of the platelet or perhaps the reason for their error may have been due to the fact that the cells in the frog which correspond to the mammalian platelet have nuclei.

Between 1882 and 1887 a series of experiments by Bizzozero (1882), Hayem (1882), Eberth and Schimmelbusch (1886) and Welch (1887) clearly demonstrated that the platelet was the first cell to appear at a site of vascular injury. These investigators examined mammalian vessels. In the latter two studies particular attention was paid to the time sequence of platelet deposition and fibrin formation. Eberth and Schimmelbusch showed that platelets were deposited first and subsequently, fibrin was formed. Welch showed that after the initial platelet deposition, as the blood flow became reduced, polymorphonuclear leucocytes and fibrin appeared on the surfaces of, but not within, the platelet aggregates. Finally, as the circulation stopped, a blood coagulum filled the spaces within the platelet deposit. Consonant with an involvement of the platelet at sites of vascular injury Lubnitzky (1885) observed areas consisting of these cells in histological sections of haemostatic plugs.

These advances in the understanding of thrombosis were made eighty years ago and they were so complete that it is only during the last decade with the application of the electron microscope, that

further advances in our knowledge of the early stages of thrombosis have been possible.

The contribution of the electron microscope

The small dimensions of platelets and their ability to spread out when placed on a hydrophilic surface probably account for the fact that these cells were one of the first studied with the electron microscope (Wolpers and Ruska, 1939). The first electron micrographs of a human thrombus do not appear to have been published until much later (Levene and Levene, 1957). They examined a pulmonary embolus and observed masses of closely packed platelets in certain areas. These platelets however were not fused, retaining their individual outlines even when their interior structure had changed markedly. This observation has been confirmed and elaborated in later studies (Poole and French, 1961; Iseri and Benditt, 1961; Honour and Russell, 1962; Florey et al., 1962; Wiener and Spiro, 1962).

This demonstration that the apparently structureless areas seen in sections of fresh thrombi examined by light microscopy do not represent fused platelets, but in fact closely packed platelets, is one of the more important findings which have emerged from the use of the electron microscope in the investigation of thrombosis. The cell separation in platelet aggregates formed in vivo is between 200 and 400 A° (Kjaerheim and Hovig, 1962). Because the limit of resolution of the light microscope is 1000 - 2000 A°, it is understandable that these platelet aggregates would appear as amorphous masses with light microscopy.

Thus many workers (Zucker, 1947; Bersagel, 1956; Zucker and Borelli, 1959; Rosenthal and Vyas, 1961; Roskam, 1961) had considered that the initial phase of thrombosis was one of platelet fusion and adhesion, rather than just adhesion. Observations with the electron microscope now indicate that this assumption is incorrect. Initially the platelets aggregate but do not fuse; thereafter the platelets may fuse (Rodman et al., 1965) but this is probably due to the process of autolysis (Zucker and Borelli, 1958; Firkin, 1963, Firkin et al. 1965). Subsequently evidence will be presented that the two substances thrombin and adenosine diphosphate may be particularly important in the initiation and growth of platelet thrombi. Employing an in vitro model, Rodman and his colleagues (1963; 1965) were able to demonstrate that thrombin is capable of inducing rhexis and lysis of the platelet whereas adenosine diphosphate had little or no effect. These workers considered the ultrastructural changes occurring in platelets during "white thrombus" formation and described four stages, namely pre-agglutination, agglutination, thrombocytorrhexis and finally thrombocytolysis.

Because of the difficulty sometimes in locating experimental thrombi produced in living animals, much of the work with the electron microscope has been with artificial thrombi. These artificial thrombi structurally have a striking resemblance to natural thrombi, but are produced under in vitro circumstances. They can be produced rapidly and consequently the material is well preserved, this being essential for adequate electron microscopy.

In 1958 A. B. Chandler showed that when blood was placed in a

closed circular loop of plastic tubing and rotated continuously on a turntable, the blood eventually solidified, and when this was examined histologically it was found to contain areas strikingly similar in appearance to a natural thrombus.

Poole, French and Cliff (1963) and Poole (1964) examined with the electron microscope both injured vessels and artificial thrombi produced in a modified Chandler apparatus (Poole, 1959), and on the basis of their observations proposed the following sequence of events in the initial stages of thrombosis.

(i) Firstly, platelets adhere to a damaged vessel wall (platelet adhesion).

(ii) Platelets then adhere to the platelets which arrived first (platelet aggregation).

(iii) The platelet aggregate changes from a loosely packed to a closely packed arrangement.

(iv) Platelet granules are discharged and a zone of degranulated platelets appears at the periphery of the aggregate. Kjaerheim and Hovig (1962) have also observed degranulated platelets in haemostatic plugs and Florey et al. (1962) noted the same in thrombotic material lining aortic fabric grafts.

(v) Leucocytes then adhere to the degranulated platelets.

(vi) At, or about this time, fibrin first appears in a recognisable form. Fibrin was not visible between the platelets in the depths of the aggregate suggesting that platelet aggregation precedes fibrin formation.

The platelet's prime function is to maintain haemostasis and structurally and biochemically this cell must be fitted for this purpose. If thrombosis can be considered as an abnormal or pathological extension of the haemostatic mechanism then any advances in our knowledge of platelet morphology must be relevant to this discussion. The electron microscope has naturally promoted a better understanding of the morphology of this cell and the various observations will presently be mentioned in the appropriate sections.

Platelet adhesion to an injured vessel wall

The seemingly well established observation that platelets accumulate at sites of vessel wall injury before visible fibrin is evident does not necessarily suggest that a mechanism unrelated to the coagulation process is involved. Platelet clumping clearly precedes fibrin formation during coagulation (Mustard and Hoeksema, 1962). This clumping is probably caused by thrombin but occurs prior to fibrin formation because platelet aggregation is a more sensitive indicator of low concentrations of thrombin than is fibrinogen clotting (Shermer et al., 1961; Mason et al., 1962). However, the speed with which platelets accumulate at points of injury appears to be inconsistent with the coagulation mechanism in a causal role. Hughes (1959a) observed the formation of platelet plugs in injured vessels within one to two seconds after initiation of the injury.

A mechanism not directly related to clotting was established with the very important observation by Hellem in 1960 that a heat-stable, protein-free, dialysable extract of human red cells could increase the adhesiveness of platelets to glass-beads. In the following year this

substance was identified as adenosine diphosphate (ADP), and it was found that clumping of platelets occurred when this substance was added to citrated platelet-rich plasma in a concentration as low as 0.02 µg per ml. (Gaarder et al., 1961). This finding promoted a new era of research into the problems of platelet adhesion and aggregation.

Gaarder and his colleagues (1961) suggested that because ADP is a naturally occurring intracellular substance, cellular damage with release of ADP may be important in the initial stages of thrombosis and haemostasis. In 1956 Born showed that platelets contain unexpectedly high concentrations of adenosine triphosphate (ATP), and he subsequently found that most of this ATP breaks down when platelets are suspended in plasma which is clotting (Born, 1958). He described a spectrophotometric method for the quantitative study of platelet aggregation (Born, 1962) and used it to investigate the effects of three phosphates of adenosine. It was confirmed that ADP causes platelets to aggregate and he suggested that the sequence of events after blood vessel injury is as follows:

> " ... ATP is broken down in damaged cells which release enough ADP to make a few platelets adhere to the damaged endothelium and to each other. At the same time tissue thromboplastin is released so that a little thrombin is formed. This brings about viscous metamorphosis in nearby platelets during which their own ATP is broken down to ADP. The ADP, whether it is on the surface of the platelets or released with their granules, causes more platelets to adhere, so that the aggregate of platelets grows

until it forms a haemostatic plug. Pathologically, the aggregation of platelets is an immediate cause of thrombosis. This aggregation may involve ADP if pathological changes cause ADP to be released from cells in the vessel walls or in the blood". (Born, 1962)

Subsequent investigations indicate that this initial phase of platelet adherence is more complex than suggested by Born. Not only do platelets adhere to damaged endothelium, but they also adhere to exposed connective tissue fibres; Roskam (1961) and Zucker and Borelli (1962) in fact concluded that connective tissue fibres are the site of platelet deposition in the traumatised blood vessel.

Numerous workers (Hugues, 1959b, 1962; Zucker and Borelli, 1962; Spaet, Cintron and Spivack, 1962; Hovig, 1963) have shown that platelets when exposed to connective tissue adhere to connective tissue fibres. Jorgensen and Borchgrevink (1964) reported that platelets normally adhere to both exposed connective tissue and to endothelial cells at the margins of the injured area in blood vessels. In von Willebrand's disease they found that the platelets did not adhere to the endothelial cells, only to the exposed connective tissue. Their observations indicate that platelets do adhere to damaged endothelium as well as to connective tissue but suggest that the mechanisms involved in each process may differ.

Nucleotides are released from injured cells and it seems not unlikely that the adherence of platelets to damaged endothelial cells could be mediated through ADP released from these cells. Caen (1963)

found a difference in the ATP content of platelet-rich plasma in patients with von Willebrand's disease and normal controls. There was a significant increase in the levels of ATP in patients with this disease, with a resultant increase in the ratio of ATP to ADP. This may be the explanation for the inability of platelets to adhere to damaged endothelial cells in this condition, in view of the fact that ATP is known to inhibit ADP-induced aggregation (Born, 1962). Vainer and Caen (1964), using a photometric technique, showed that small amounts of ADP, active in normal subjects, failed to induce platelet aggregation in subjects with von Willebrand's disease. With larger amounts of ADP however, the responses were quantitatively the same. It may be that in von Willebrand's disease the small amount of ADP released from injured endothelial cells is insufficient to promote adherence of platelets to these cells in the presence of the elevated level of ATP. Ödegaard, Skalhegg and Hellem (1964a) have also demonstrated that ADP-induced platelet adhesiveness to glass is impaired in von Willebrand's disease.

Using a traumatised mesentery preparation and phase microscopy, Spaet and Zucker (1964) showed that platelet adhesion to connective tissue fibres is independent of divalent cation concentrations, is not altered when platelets are made less electronegative, and unlike ADP-induced platelet aggregation is not inhibited by adenosine monophosphate (AMP). Moreover, thrombasthenic platelets did adhere to connective tissue but were incapable of aggregation. For these reasons it is highly unlikely that the platelet-connective tissue reaction is mediated by the ADP mechanism.

In an interesting study on platelet adhesiveness and aggregation using latex particles, Glynn, Movat, Murphy and Mustard (1965) demonstrated the presence of these particles in vacuoles inside the platelet suggesting that platelets can phagocytose latex particles. They pointed out that "it has not been generally recognised that platelets have phagocytic activity". David-Ferreira (1964) and Schulz (1961) showed that platelets take up thorotrast, and Haguenau and his associates (1963) that platelets take up ferritin. Danon, Jerushalmy and DeVries (1959) also reported the occurrence of virus particles inside platelet vacuoles. Polymorphonuclear leucocytes are known to ingest latex particles and it is thought that this is an energy-dependent process (Robert and Quastel, 1963). According to Allison, Lancaster and Crosthwaite (1963) white cell phagocytosis involves activation of a proteolytic enzyme or enzymes influencing the cell membrane. Movat, Weiser, Glynn and Mustard (1965) showed that the latex-particle platelet reaction can be inhibited by concentrations of epsilon aminocaproic acid that have been found to inhibit phagocytosis by white cells, and suggested that this evidence could mean that the initial adherence of platelets to a surface involves activation of a proteolytic enzyme. They suggested that the reaction of platelets with collagen fibres and glass surfaces is similar to particles which can be ingested, except that instead of phagocytosis the platelet spreads over the larger surfaces.

Sawyer (1965) states that the "surface charge of the blood vessel wall, as elucidated by findings among a substantial group of investigators, must be considered a major homeostatic mechanism,

TABLE 1 (cont.)

4. MISCELLANEOUS	
Thrombin	O'Brien (1962a, 1963b) Clayton and Cross (1963) Haslam (1964)
5-hydroxytryptamine	O'Brien (1964) Mitchell and Sharp (1964)
Collagen	Zucker and Borrelli (1962) Hovig (1963)
Triethyltin	O'Brien (1963a)
Nicotine	Werle and Schievelbein (1965)
Oxime esters	Constantine and Hochstein (1966)
Latex particles	Glynn et al. (1965)
Bacterial endotoxin	Ream et al. (1965)
Staphylococcal toxins	Jeljaszewicz et al. (1966)
Dextrans	Dhall et al. (1966)
Antigen-antibody complexes	Movat et al. (1965a)
Snake venoms	Davey and Luscher (1965)
Neuraminidase	Hovig (1965)
Kaolin	Hardistry and Hutton (1966)
Methyl mercuric nitrate	Robinson et al. (1965)
Trypsin	Haslam (1964)

TABLE 1

COMPOUNDS PRODUCING IN VITRO PLATELET AGGREGATION

CATEGORY OF COMPOUND	REFERENCE
1. NUCLEOTIDES	
Adenosine diphosphate	Gaarder et al. (1961) Born (1962)
Deoxy adenosine diphosphate	Gaarder et al. (1961)
3-deoxy adenosine diphosphate	Gaarder and Laland (1964)
Adenosine diphosphate 1-N-oxide	Gaarder and Laland (1964)
Adenosine triphosphate	Mitchell and Sharp (1964)
Adenosine tetraphosphate	Clayton et al. (1963)
2. CATECHOLAMINES	
Adrenaline	O'Brien (1963b) Clayton and Cross (1963) Mitchell and Sharp (1964)
Nor-adrenaline	O'Brien (1964, 1963b) Clayton and Cross (1963) Mitchell and Sharp (1964)
3. LIPIDS	
Sodium lignocerate	Haslam (1964)
Sodium behenate Sodium arachidate Sodium stearate Sodium palmitate	Haslam (1964) and Mahadevan et al. (1966)
Sodium myristate Sodium laurate	Mahadevan et al. (1966)
Phosphatidic acid Phosphatidyl serine Phosphatidyl ethanolamine Sphingomyelin and lysolecithin Lecithin stearate sol. Lecithin palmitate sol. Lecithin myristate sol. Lecithin oleate sol.	Kerr et al. (1965)

normally preventing abnormal thrombosis". Conversely, in the event of injury he suggests that it may also be a source of vascular thrombosis. Perhaps this accounts for the attraction of platelets to connective tissue fibres although Spaet and Zucker (1964) did show that platelet adhesion to connective tissue fibres is not altered when platelets are made less electronegative.

To summarise our present knowledge of the initial stages of thrombosis, it appears that at a site of vascular injury, platelets adhere to damaged endothelial cells and to subendothelial connective tissue fibres. Platelets adhere rapidly before fibrin is visible. Platelet adherence to damaged endothelial cells may be mediated by ADP. While adherence to connective tissue fibres remains largely unexplained, there is evidence that ADP may not, in fact, be implicated, and a phagocytic response involving activation of a proteolytic enzyme has been suggested.

Platelet aggregation

As well as their ability to stick to other surfaces such as collagen, platelets also have the ability to stick to each other and this is important in the earliest stages of haemostasis and thrombosis. There has been intense interest in the subject of platelet-to-platelet adhesion or platelet aggregation since the observation by Gaarder and his colleagues (1961) that ADP induces clumping of platelets. The list of substances able to induce platelet aggregation is now considerable and these are detailed in Table I. Of these many substances, ADP and thrombin are two that may both be involved in the development of the platelet plug

or aggregate associated with vascular injury.

Whole thickness aorta and aortic luminal scrapings both contain a heat-stable substance which has clumping activity (Mitchell and Sharp, 1964), and which is probably ADP. This nucleotide is probably released from injured cells in the vessel wall (Gaarder et al., 1961; Born, 1962) and initially this may cause a few platelets to adhere to each other. Zucker and Borelli (1962) showed that platelet clumps attached to collagen fibres developed within 1 to 2 minutes after the introduction of isolated collagen fibres into platelet-rich plasma; this aggregation is due to the release of ADP from platelets adherent to the collagen (Hovig, 1963; Holmsen, 1965). The release of ADP from platelets which are adhering to denuded collagen exposed by vascular injury could be a fundamental and significant mechanism in promoting the growth of platelet clumps at such sites; this mechanism could well be more important than the release of nucleotide from injured cells of the vessel wall or from damaged erythrocytes (Hellem, 1960).

Several studies have shown that blood vessels also possess tissue thromboplastic activity (Astrup, et al., 1959; Kirk, 1960; Donner, 1962; Prentice, McNicol and Douglas, 1966). As a result of injury this tissue thromboplastin may also be released, with consequent formation of thrombin. Therefore after a vascular injury both ADP, released from injured cells in the vessel wall, from erythrocytes, and from platelets adherent to collagen, and thrombin, formed due to release of tissue thromboplastin, could initiate the formation of a platelet plug. Thrombin also releases 5-hydroxytryptamine from

platelets (Davis and Palmer, 1965), and this is another substance known to be capable of causing platelet aggregation (Mitchell and Sharp, 1964; O'Brien, 1964). Just recently, Niewiarowski and Thomas (1966) demonstrated a synergistic effect of ADP and thrombin in platelet aggregation; this could have important implications in haemostasis and thrombosis.

This concept of the events immediately following vascular injury is based largely on in vitro observations, but it has been tested in vivo by Honour and Mitchell (1964) and Born, Honour and Mitchell (1964). These workers claimed that in injured rabbit cerebral cortical arteries, the formation of platelet thrombi was enhanced by ADP. Furthermore, the infusion of the nucleotides adenosine and 2-chloro adenosine, known to inhibit ADP clumping in vitro (Table 2) prevented the formation of the usual platelet mass at the injury site.

Mechanism of thrombin-induced aggregation

Although it has been proposed that thrombin clumping activity is mediated through conversion of fibrinogen on the platelet membrane to fibrin, which then binds the platelets together (Apitz, 1939; Schmid, Jackson and Conley, 1962), the following observations argue against this suggestion. Electron microscopic examination of thrombin-induced platelet aggregates has failed to demonstrate any fibrin between the platelet membranes (Kjaerheim and Hovig, 1962; Rodman, Mason and Brinkhous, 1963). Secondly, small amounts of thrombin which will not clot fibrinogen in two minutes will aggregate platelets in about eighteen seconds (Mason et al., 1962). Thirdly, afibrinogenaemic

platelets have been reported to aggregate normally with thrombin (Gugler and Luscher, 1965).

Kaser-Glanzmann and Luscher (1962) and Grette (1962) showed that ADP is released from platelets acted upon by thrombin and the former workers suggested that thrombin activity is mediated via this released ADP. Several observations strongly suggest that thrombin acts through an intrinsic ADP system, the ADP being derived from platelet ATP. There is a delay of several seconds before aggregation commences (O'Brien, 1964) and the pyruvate kinase and phosphoenol-pyruvate system which converts ADP to ATP (Haslam, 1964) and adenosine (Born and Cross, 1963) and AMP (Born, 1962) which are all known to interfere with ADP-induced platelet aggregation, all inhibit thrombin clumping activity. Thrombin causes the platelet granules to disappear (Vassalli et al., 1964) and this may relate to the release of ADP, serotonin and other substances from platelets.

Mechanism of ADP-induced aggregation

At physiological pH the blood cells are all negatively charged, and are, on consideration of overall charge alone, able to remain discrete. (Bangham et al., 1958). Pethica (1961) listed nine kinds of attraction forces between cell membranes. The list included chemical bonds, the formation of ion pairs including calcium bridges, charge mosaics, surface tension and van der Waals' forces. The relevance of many of these forces to biological adhesion remains obscure.

Various hypotheses have been proposed to explain the phenomenon

of platelet aggregation, the only feature common to each theory being that calcium or other divalent cations are involved in electrostatic bridging between platelets. There is little doubt that ADP-induced clumping of platelets is cation dependent (Born and Cross, 1963a; Mitchell and Sharp, 1964). Other properties which have to be taken into account in any explanation proposed to explain the activity of ADP are the rapidity of its action and its highly specific effect, closely related compounds being totally inactive (Born, 1962) and the finding that on the addition of ADP, the platelet granules persist (Sokal, 1963; Rodman, Mason and Brinkhous, 1963).

(i) Mitchell and Sharp (1964), by studying the inhibitory effect on platelet aggregation of various agents which they assumed to be selectively active either on the platelet surface or on the platelet content, concluded that clumping substances act at the platelet surface. ADP, ATP, nor-adrenaline and 5-hydroxy-tryptamine are substances which the platelets normally contain, and they suggested largely on theoretical grounds that if a substance normally present within the platelet is bound to its external surface, the membrane potential due to the gradient of this substance would be abolished, with consequent alteration of the surface charge. According to Bangham (1962) an approach velocity of 2 cm. per second or greater is required for platelets to overcome mutual repulsion. With alteration of the surface charge, however, lower approach velocities would suffice.

(ii) Skalhegg, Hellem and Odegaard (1964) and Gaarder and Laland

(1964) suggested that the ADP molecule is bound to the platelet surface and aggregation results from a participation of this molecule in an inter-cellular bridge. Two nucleotides known to bring about aggregation, namely ADP and adenosine tetraphosphate (Clayton et al., 1963) have at physiological pH an uneven number of negative charges, whereas the inhibitors have no charge at all (adenosine) or an even number of charges (AMP and ATP). These workers therefore proposed that the adenine part of the nucleotide molecule attaches to protein located on the surface of the platelet by hydrogen bonding; calcium ions then complete the bridge by attaching to the odd negatively charged group remaining with ADP or adenosine tetraphosphate. The nucleotides which exhibit no clumping activity would have their even number of valences neutralized by the two positive valences of calcium ions and would therefore be unable to participate in a bridge.

However, the observation that ADP causes very rapid swelling of platelets, even before they become sticky (O'Brien, 1965; Bull and Zucker, 1965; O'Brien and Heywood, 1966) suggests that ADP has a metabolic action, rather than participating in bridge formation as espoused above. The usual shape of the platelets in the body is an oval flattened disc and energy will be required to maintain this configuration. The swollen rounded platelet is the configuration requiring least energy to maintain. The following interesting hypothesis attempts to explain ADP-induced aggregation on a metabolic basis. This is the

most plausible explanation to date.

(iii)Platelet membrane ATP-ase is responsible for maintaining the cell in its normal size and shape. Inhibition of this ATP-ase by the product of its normal action, namely ADP, leads to sphering of the platelet with consequent exposure of receptive sites. These exposed sites on the platelet surface then permit secondary bridging (Salzman, Chambers and Neri, 1966a, b). Spaet and Lejnieks (1966) also proposed an energy-yielding reaction to explain clumping. They however suggested that the breakdown of ADP into AMP releases energy to be made available for platelet binding reactions. The breakdown of ADP to AMP is blocked by potassium cyanide (Salzman, Chambers and Neri (1966b) but this in fact facilitates aggregation, thus making the hypothesis of Spaet and Lejnieks untenable. Consistent with a metabolic hypothesis, aggregation has been found to be temperature dependent, occurring most quickly at 37°C, being slower at 20°C and not detectable at 4°C (O'Brien, 1966a). Furthermore, mono-iodoacetate which blocks glycolysis, the most important metabolic pathway in platelets (Gross, 1961), inhibits ADP-induced platelet adhesiveness (Skalhegg et al., 1964) and modifies aggregation (O'Brien, 1966).

A plasma co-factor also appears to be involved in ADP-induced platelet aggregation. Platelets suspended in a solution of inorganic ions will not clump in the absence of platelet-free plasma (Born and Cross, 1963a, 1964; McLean, Maxwell and Hertler, 1964). Odegaard and his colleagues (1964) suggested that the plasma factor required is

that lacking in von Willebrand's disease, but this seems unlikely because Cross (1964) has reported a normal response to ADP in patients with this condition. The evidence to date indicates that fibrinogen may be a plasma co-factor (Cross, 1964; McLean et al., 1964; Brinkhous et al., 1965; Inceman et al., 1966). Deykin, Pritzker and Scolnick (1965) concluded that two plasma factors, fibrinogen and a heat-stable plasma protein, may participate in ADP-induced platelet aggregation.

According to Laki and Gladner (1964) fibrinogen contains neuraminic acid which can be split off in the free form by treatment with neuraminidase. Platelets treated with neuraminidase aggregate spontaneously when resuspended in plasma (Hovig, 1965). This finding, and the observation that the proteolytic enzyme trypsin destroys the ability of platelets to aggregate with ADP provide further indirect evidence of the involvement of fibrinogen in platelet aggregation. In conclusion, a plasma co-factor(s) is undoubtedly a requisite for platelet clumping, but its precise nature and mode of involvement requires further definition.

Inhibition of ADP-induced aggregation

Although it has not yet been established whether the ADP mechanism has a primary role in thrombosis, the implication of ADP in aggregation by other agents such as thrombin and fatty acids (Haslam, 1964), epinephrine (O'Brien, 1964) and collagen (Hovig, 1963) ensures that it must almost certainly in some way be involved in thrombosis. Therefore compounds which modify or inhibit the activity of ADP might be of value in the treatment of thrombosis.

Following the observation that adenosine monophosphate (AMP)

TABLE 2 (cont.)

INHIBITOR	REFERENCE
Fibrinogen degradation products	Kowalski et al. (1964)
Phentolamine	O'Brien (1963b)
Phenoxybenzamine	Clayton and Cross (1963)
Dichloro-isopropylnoradrenaline	Clayton and Cross (1963)
Heparin	O'Brien (1963b) Clayton and Cross (1963)
Lecithin sols of some unsaturated fatty acids	Kerr et al. (1965)
Sulphydryl inhibitors: N-ethyl maleimide P-hydroxymercuribenzoate	Harrison et al. (1966)
Methyl mercuric nitrate	Robinson et al. (1965)
5HT antagonists	Mitchell and Sharp (1964)
Trypsin	Haslam (1964)
Phosphoenolpyruvate plus pyruvate kinase	Haslam (1964)

TABLE 2

INHIBITORS OF PLATELET AGGREGATION IN VITRO

INHIBITOR	REFERENCE
Antihistaminics, antimalarials and local anaesthetics	O'Brien (1962a)
Adenosine monophosphate	Born (1962)
Adenosine triphosphate	Born (1962)
Adenosine	Born and Cross (1963)
Adenosine analogues: 2-Chloro-adenosine 2-Bromo-adenosine 2-Fluoro-adenosine 2-Aza-adenosine	Born et al. (1965)
Adenosine 1-N-oxide	Born et al. (1965)
EDTA	Born and Cross (1963)
Toluidine blue	Mitchell and Sharp (1964)
Promethazine hydrochloride	Mitchell and Sharp (1964)
Histamine	Constantine (1965)
Substituted amino acids: Arginine methyl ester Benzoylarginine methyl ester Benzoylarginine ethyl ester Tosylarginine methyl ester and others	Salzman and Chambers (1964)
Dipyridamole	Emmons et al. (1965)
Urea	Hellem et al. (1964)
Sodium cyanide plus sodium iodoacetate	O'Brien (1966)

inhibits aggregation by ADP (Born, 1962), an intensive investigation of the properties of this inhibition ensued, and a formidable number of other inhibitory substances were discovered (Table 2). Adenosine (Born and Cross, 1963) and several of its analogues (Born et al., 1965) are more effective inhibitors of ADP than is AMP and are far more powerful than certain antihistaminics and local anaesthetics (O'Brien, 1962a) and the esters of some amino acids (Salzman and Chambers, 1964).

The intravascular injection of ADP into several species including man (Davey and Lander, 1964), cat (Born and Cross, 1963b) and the rabbit (Regoli and Clark, 1963), induces a fall in the circulating platelet count. Silver and Stehbens (1965), using a transparent rabbit ear chamber preparation showed that this fall in platelet count was due to the formation of platelet aggregates which were then trapped in small vessels. Aggregation is rapidly followed by dis-aggregation and the platelet count consequently rises again. It has been suggested that both platelet and plasma enzymes (Kerby and Taylor, 1964; Rutty, 1965; Turtle and Firkin, 1965; Born, 1966) break ADP down to AMP, adenosine, and other derivitives which then antagonise ADP aggregation. Regoli and Clark (1963) showed that adenosine inhibition does work in vivo. This would also explain the statement by Sharp (1965) that there "is a limit to the size of platelet clumps in any given experimental system ...."

It has already been mentioned that adenosine and 2-chloroadenosine inhibit the formation of platelet thrombi in the lumen of an injured vessel

(Born et al., 1964), and fortunately without apparently interfering with haemostasis. Unfortunately, however, adenosine (and its analogues) proved to be a powerful vasodilator, producing a marked fall in blood pressure (Born et al., 1965). Moreover it is rapidly broken down in the body by deamination, and although 2-chloroadenosine is more resistant to deamination, this latter compound produced respiratory arrest in rabbits (Born et al., 1964). A compound which might, however, prove to be of value in the treatment of thrombosis is dipyridamole. This slows the rate of disappearance of exogenous adenosine from whole blood (Bunag et al., 1964), and is a powerful inhibitor of thrombus formation in injured rabbit vessels (Emmons et al., 1965a). It inhibited ADP-induced aggregation in vitro and when injected intravenously into humans proved to be free of side effects and reduced the amount of spontaneous clumping (Emmons et al., 1965). These workers suggested that clinical trials of dipyridamole in occlusive vascular disease in man should be undertaken. The treatment of thrombosis can never be a very satisfactory approach, however, and the more important aim must be to find the cause and thereby prevent this condition.

Stabilisation and growth of the platelet aggregate

Evidence has been presented that at least two of the clotting factors, thrombin and fibrinogen are involved in the early stages of platelet clumping in injured vessels. However this phase appears to be independent of fibrin formation. Subsequently, the stabilisation and growth of the platelet thrombus are dependent

on the chain of biochemical reactions leading to fibrin formation.

The haemostatic platelet aggregate in normal persons has a layer of fibrin around the periphery (Jorgensen and Borchgrevink, 1963a,b). The importance of this perimetric layer of fibrin in the stabilisation of the aggregate is obvious when clotting defects are considered. In haemophilia, hypoproconvertinaemia, and with anticoagulant therapy, fibrin formation is impaired and the platelet plugs or aggregates are permeable, friable and easily dislodged (Jorgensen and Borchgrevink, 1964). An intact clotting system is therefore necessary to stabilise the platelet aggregate in haemostasis. The extrinsic and the intrinsic clotting systems both participate in haemostasis. While the intrinsic mechanism can compensate for defects in the extrinsic coagulation mechanism, the extrinsic system cannot compensate for defects in the intrinsic system (Jorgensen and Borchgrevink, 1964).

Fulton, Akers and Lutz (1953) and Honour and Russell (1962) claimed in the experimental animal that anticoagulant therapy produces unstable platelet aggregates. Intensive heparin therapy however, did not prevent platelet adherence or aggregation at the site of injury, which again confirms the conclusion that fibrin is not implicated in the earliest stages of thrombosis. Streptokinase, through its fibrinolytic effect can also interfere with the stability of haemostatic plugs (Hirsh et al., 1966).

In the presence of an adequate clotting mechanism, fibrinogen in the plasma surrounding the platelet aggregate will be converted to

fibrin when the rate of thrombin formation is sufficient to overcome the effect of dilution by flowing blood and neutralisation by antithrombin (Monkhouse, France, and Seegers, 1955).

Because it releases platelet ADP and phospholipid (Kaser-Glanzmann and Luscher, 1962) and 5-hydroxytryptamine (Grette, 1962; Davis and Palmer, 1965) thrombin is probably important in determining the growth of thrombi. The released ADP and 5-hydroxytryptamine could produce further aggregation, and the released phospholipid, by activating prothrombin, will produce further amounts of thrombin. If the thrombin level is sufficiently reduced by adsorption to fibrin (Seegers, Nieft and Loomis, 1945) neutralisation by antithrombin, and dilution by flowing blood, the thrombus will not manifest further growth. However if the blood flow is disturbed with eddy formation or stasis, thrombin dilution and neutralisation may be impaired. In flowing blood further platelet deposition would then occur with another perimetric layer of fibrin. A repetition of this process could account for the laminated appearance, so well known in arterial thrombi. In a slow or arrested blood stream, for instance in the venous circulation, larger amounts of thrombin would be available giving rise to more extensive fibrin formation; hence the red tail of a venous thrombus, which morphologically is a blood coagulum. Blood flow is obviously of vital importance in determining the structure of a thrombus (Wessler et al., 1959; Downie et al., 1963).

Jorgensen and Borchgrevink (1964) have further suggested that

in addition to the perimetric layer of fibrin necessary for the solidification and permanency of the plug, an incipient clotting on the platelet surface within the aggregate is essential to the stabilisation of this aggregate shortly after the platelets have been arrested. In haemophilia, in contrast to normal subjects, the aggregates were loose, and the platelets did not show balloon formation. Thrombin may be the factor involved in this process of stabilisation, for it has been shown that this substance will produce a swelling or ballooning of platelets (Bull and Zucker, 1965; O'Brien, 1965). The incipient clotting envisaged by Jorgensen and Borchgrevink does not proceed right through to fibrin formation, because it has been widely reported that fibrin is not present within the depths of platelet aggregates. This could be explained by the amount of thrombin formed and the observation that platelet aggregation is a more sensitive indicator of low concentrations of thrombin that is fibrinogen clotting (Mason et al., 1962).

Thrombin formation is therefore obviously important in the early stages of thrombosis. Released tissue thromboplastin will produce thrombin, but other mechanisms may also be involved in the early formation of this substance. Indeed, platelet aggregation by ADP is associated with acceleration of clotting. This acceleration does not occur when ADP is added to platelet-poor plasma, and it therefore appears that it is due to an effect on the platelet. Moreover, it seemed to be proportional to the amount of aggregation which took place (Mustard et al., 1964a). These workers suggested

that ADP may cause a release of platelet clotting activity, probably platelet Factor 3. The mechanism whereby the lipid becomes available to the coagulation process could theoretically be explained by the observations of Iatridis, Ferguson and Iatridis (1964). Their results indicated that Factor XII which is in the "plasmatic atmosphere" of platelets, may be activated when the platelet surface is altered. That is, the altered platelets provide the surface for activation of the Hageman factor, the first phase in the clotting mechanism. ADP produces swelling of platelets (Bull and Zucker, 1965) and this may provide the necessary surface alteration for activation of the clotting process; also platelet micelles might be released through the altered cell membrane as suggested by White and Krivit (1966). ADP-induced aggregation itself might therefore give rise to thrombin formation.

Thrombosis and the atherosclerotic plaque

The knowledge that platelets do adhere to damaged vessel walls raises two questions. Firstly, what constitutes an injury to the vessel wall, and secondly, is there any definite evidence of vascular damage causing thrombosis in man? A biochemical injury in the absence of demonstrable histological damage may prove to be sufficient to induce thrombosis. This aspect will be considered subsequently.

Coronary thrombosis in man almost invariably occurs in atherosclerotic arteries (Gore et al., 1960; Strong and McGill, 1962); occlusive coronary artery thrombi can occur without discernible arterial wall pathology (Matz et al., 1964). However, the mechanism responsible for the initiation of thrombosis in atherosclerotic vessels

remains open to speculation. The fact that occlusive thrombi are most frequently seen with stenotic lesions (Crawford, Dexter and Teare, 1961; Mitchell and Schwartz, 1963) is, according to Mustard and his colleagues (1964), consistent with the known effects of an alteration in blood flow associated with stenotic lesions and perhaps also to a disturbance of the metabolism of the vessel wall in the manner suggested by Sawyer et al. (1964).

Although a few investigators in the past have studied thrombosed coronary arteries by a serial section technique, those who have attempted such a study are far from agreement in respect to their interpretation of the phenomena observed. There are those who think that coronary thrombosis may be caused by fissures in the surface of the atheromatous plaque (Leary, 1934; Clark, Graef and Chasis, 1936; Drury, 1954; Constantinides, 1964) while others have concluded that an intramural haemorrhage in some way initiates the process (Paterson, 1936, 1938; Wartman, 1938; Winternitz, Thomas and LeCompte, 1938; Horn and Finkelstein, 1940). The relationship has since been clarified in three independent studies (Chapman, 1965; Friedman and Van den Bovenkamp, 1966a,b; Constantinides, 1966). From the results of these serial section studies it now appears that a fracture of the surface of an atherosclerotic plaque is the primary event and this allows a communication between the blood in the lumen of the vessel and the contents of the atheromatous plaque. This communication precedes and induces thrombosis. This temporal sequence was suggested by the presence of atheromatous gruel in the

thrombi and the direction of the fragmented ends of the ruptured wall. The author has confirmed these observations in random instances. Fissures and aortic thrombosis have been produced experimentally in mammalian arteriosclerotic arteries by a combination of acute hypertension and the injection of Russell's viper venom (Constantinides and Lawder, 1963). The factors producing fissures in human plaques remain to be determined.

Thus there is mounting evidence that injury to the vessel wall may be an extremely important factor in determining the onset of occlusive coronary thrombosis and therefore being a significant factor in one of the major causes of human mortality.

(2) The composition of the blood and thrombosis

So far, the sequence of events in thrombosis following obvious vessel injury have been presented. Under these circumstances the very early stages would seem, on the evidence available, to be independent of the quality of the blood. Can thrombosis, however, occur in the absence of preceding wall disease, and be fostered primarily by a change in the blood? Clinical experience indicates that there may be a thrombotic tendency in several disease processes. For instance, there seems to be an abnormality in the circulating blood in some young persons with occlusive arterial disease, in post-operative and post partum states, in subjects with diabetes mellitus, and in certain patients with widespread venous thrombosis.

A generalised change in the circulating blood

It is well established that patients with myocardial infarcts have, as a group, more severe coronary stenosis than patients without infarcts. This fact is clearly documented in Mitchell and Schwartz's splendid monograph on arterial disease (1965). This finding has led to the widely held belief that it is an excessive amount of wall disease which produces thrombosis in these patients. Mitchell and Schwartz (1965) however, offer an equally plausible explanation for this relationship between wall disease and thrombosis, based on their meticulous necropsy examinations. They propose some primary alteration in the circulating blood, with resultant formation of thrombi at certain sites in the vascular tree, these sites being determined by blood-flow patterns. These thrombi then organise into wall plaques, as previously suggested by Rokitansky (1852) and Duguid (1946). A repetition of this process over several years will result in severe wall disease; at this stage, when the collateral reserve has been considerably reduced, an occlusive thrombus may then cause a myocardial infarct. Hence a relationship between severe degrees of coronary narrowing and thrombosis, which initially at least, may not have been causal, and which may be related to a change in the circulating blood.

Mitchell and Schwartz (1965) also provide another fragment of evidence in support of a generalised change in the circulating blood. In Great Britain during the second world war, no change in the number of deaths attributed to pulmonary embolism was observed, but

thereafter a striking and progressive increase in the number of deaths was recorded. (The death rates were obtained from the Registrar-General's Statistical Review of England and Wales for the period 1940-1958). There is an obvious resemblance between this trend and that for myocardial infarction. These observations suggest an increasing tendency to thrombosis in both veins and arteries, that is, a generalised change, and if real, an environmental factor may be the cause.

Evidence that vascular injury is a concomitant of venous thrombosis is sparse, again suggesting that we should focus our attention upon the circulating blood (or upon irregularities in blood flow). If veins are examined by orthodox techniques of sectioning and staining, no microscopic changes are demonstrable (Paterson and McLachlin, 1954). However, when the endothelial lining was examined by staining the cement substance, O'Neill (1947), Samuels and Webster (1952), and McGovern (1955) claimed that areas of abnormal endothelium could be found and concluded that endothelial damage might be the basic lesion in venous thrombosis. Whether such changes are important in the genesis of venous thrombosis remains unresolved.

The generalised change in the circulating blood indicated by the foregoing discussion may arise in two ways. Firstly, an alteration in the coagulation mechanism may be responsible or secondly, there may be a primary change in the circulating platelets, these cells manifesting an increased "stickiness".

The evidence for these two proposals is provided by the observations presented herein.

Blood coagulation and its relation to thrombosis

By placing blood in a rotating tube thereby imitating blood flow within a vessel, Chandler (1958) and Poole (1959) demonstrated that the solid structure which formed was not entirely homogeneous as is a typical clot. Platelet aggregates and the other usual morphological features of a thrombus were visible in the "head" of this structure. Platelet clumping clearly precedes fibrin formation in thrombosis but the same sequence of events also occurs in clotting (Mustard and Hoeksema, 1962). Thus in some circumstances the distinction between a clot and a thrombus may be spurious. The same sequence of events is present in the development of both, and the different morphological characteristics are merely due to the conditions under which they form. Only when there is blood flow, will there be significant platelet aggregation and the development of the orientated features of a thrombus. The factor which causes platelet changes including clumping in the coagulation process is probably thrombin (Mustard and Hoeksema, 1962; Fantl and Sweet, 1966).

There is considerable experimental evidence to indicate that stimulation of the coagulation mechanism can initiate thrombosis. McLetchie (1952) and Mitchell (1964) showed that the injection of Russell's viper venom into rabbits produced widespread thrombi. These thrombi contained typical platelet aggregates. This venom contains an enzyme capable of activating Factor X (Macfarlane, 1961;

Williams and Esnouf, 1962), thereby initiating clotting and producing thrombin. It has been shown that thrombin *in vivo* will produce platelet clumping, and moreover that this occurs before fibrin is formed (Vassalli *et al.*, 1964).

There are also other sites in the coagulation sequence at which activated coagulation moieties will result in thrombosis in experimental animals. Infused tissue thromboplastin will also produce thrombi (Mason, 1924; Penick *et al.*, 1958; Spaet and Kropatkin, 1958; Vassalli *et al.*, 1964). Finally the infusion of substances that activate the endogenous precursors of intrinsic or blood thromboplastin will produce thrombi (Wessler, 1963). With this latter mechanism stasis is required except when an extracorporeal shunt is included in the circulation and then a platelet rather than a red thrombus is elaborated (Downie *et al.*, 1963). Wessler (1955) produced stasis thrombi by injecting normal human serum into a mammalian recipient, and it has since been shown (Henderson and Rapaport, 1962; Deykin and Wessler, 1964) that activated Factor IX, through activation of the Hageman factor, is the factor in the serum responsible for inducing thrombosis in areas of vascular stasis. In freely flowing blood, activated clotting intermediates are probably removed by dilution and by inhibitors (Nossel, 1964), but in areas of retarded blood flow these effects would be reduced. Because these thrombi only develop in areas of stasis, structurally they have the homogeneous nature of a clot - for this reason many workers have dismissed stasis thrombi as artefacts. However,

venous thrombi may have a large homogeneous component and in the slower venous circulation it is conceivable that activation of the clotting mechanism may play a role in the pathogenesis of thrombosis.

The concept of hypercoagulability

Alexander (1962) in a review devoted to the relationship between blood coagulation and thrombotic disease expresses the popular belief that thrombosis and haemorrhagic diatheses may be opposite processes. In the haemorrhagic disorders, delayed clotting is associated with a quantitative or qualitative deficiency in one or more clotting factors, or with circulating natural or administered anticoagulants in excess, and it has been widely assumed therefore that in some instances at least, "intravascular clotting might be due to excessive amounts or activity of one or more of the procoagulant factors. Similarly, a decrease in anticoagulant factors is also possible". The term "hypercoagulability" has been applied to this abnormally increased activity of blood coagulation in which an increased risk of developing thrombosis is implied.

Clinically, there are a few conditions in which there is circumstantial evidence that an increased activity of blood coagulation may occur in man. Venous thrombosis is a well recognised association of visceral cancer, especially carcinoma of the pancreas (Kenney, 1943; McKay, Mansell and Hertig, 1953). No obvious disease of the vessel wall is apparent although a chemical change in the wall cannot be exempt, and it has therefore been suggested that the clotting mechanism may be

activated by foreign material in the blood stream (Sise, Moschos and Becker, 1962). In accord with an activated clotting mechanism, Ingram (1954) has stated that the prothrombin time is significantly shorter in some cases, and Goodman (1958) has described a patient with carcinoma of the pancreas in which there was inappropriate haemorrhage in response to a dose of anticoagulant which he suggested might be due to a rapid turnover of clotting factors.

Kosrewski and Vahabzadeh (1964) reported the occurrence of widespread multiple arterial thrombosis in the systemic and pulmonary circulation of a thirty five year old male in which there was a marked resistance to heparin. Laboratory studies indicated that the patient's plasma was devoid of heparin co-factor. This case represents an increased clotting tendency due to a decrease in coagulation inhibitor activity. Admittedly this is an isolated report, but it does suggest that a state of hypercoagulability can occur in man.

Egeberg (1966) commented on two Norwegian families in which there was a high incidence of thrombo-embolic diseases associated with abnormalities in blood coagulation. Affected members in one family had a deficiency in blood antithrombin III and in heparin co-factor (antithrombin II). In the other family, the thrombotic tendency seemed to be due to an inherited abnormality in which the fibrinogen was abnormally sensitive to thrombin.

These few seemingly bizarre reports represent the only definite examples linking thrombosis with a primary alteration in the blood coagulation mechanism, and illustrate how scanty and

indefinite is our knowledge of the role of the coagulation mechanism in thrombosis. If thrombosis is a primary factor in the pathogenesis of atherosclerosis in man (Duguid, 1946), then a generalised change in the circulating blood may well exist, and in view of the facts just present, an alteration in the blood coagulation mechanism producing thrombosis has to be considered. This possibility has, in fact, been extensively investigated in the laboratory.

Changes in the blood in the direction of hypercoagulability can be grouped and discussed as follows:

(i) Increase in the amount or activity of procoagulant factors

On the assumption that intravascular clotting might be due to excessive amounts of procoagulant factors, many workers have claimed to have demonstrated a hypercoagulable state in various clinical conditions known to be associated with a thrombotic tendency. The concentration of Factor VIII may be increased in the post-operative period (Egeberg, 1962; Amundsen et al., 1963), in pregnancy (Jorpes and Ramgren, 1962; Strauss and Diamond, 1963) and in ischaemic heart disease (Cooperberg and Teitelbaum, 1961). In pregnancy, Factor IX (Ratnoff and Holland, 1959), Factor VII (Alexander et al., 1956) and Factor X (Pechet and Alexander, 1961) have also been reported to be elevated.

A consistent correlation between an increase in the amounts of procoagulant factors and thrombosis has not been established, however, other investigators having found normal and even

decreased yields of these factors. The reasons for these discrepancies may be in part technical, but probably more important is the time at which the assays have been performed in relation to the stage of thrombosis. Experimentally, when the rate of intravascular coagulation is increased by supplying artificial surface activation, there is an associated decrease in the level of Factor VIII due to its utilisation. Contrariwise, a decrease in the rate of intravascular clotting after anticoagulant therapy is associated with an increase in the level of Factor VIII (Sise et al., 1962). Thus the results obtained when estimating the level of this factor, and probably other factors, will depend on whether the tests are being performed prior to, during, or after the thrombotic event. Thrombosis can occur without being clinically manifest, and therefore correlation of the level of plasma coagulation factors with the stage of thrombosis is impossible. An increase in coagulation factors may prove to be the result of a thrombotic event and not the cause. Moreover, all of the clotting factors are already present in excess in the normal person (Alexander, 1962; Sise et al., 1962).

In recent years it has been claimed that there is an association between the use of steroid oral contraceptive drugs and thrombo-embolic episodes (Reed and Coon, 1963; Nevin et al., 1965). The report that these drugs produce an increase in the blood levels of Factors VII and VIII (Egeberg and Owren, 1963) and fibrinogen (Brakman and Astrup, 1964) once again

raises for consideration the effect of high levels of clotting factors on the coagulability of blood.

The clotting system is very complex and because only one end point is available many tests are required to fully study the process. Some of these tests are specific for the activity of a particular clotting factor, but others give information about the process in part or in whole. Clotting tests designed to give information about a part or the whole of the coagulation mechanism have also been performed in conditions associated with a thrombotic tendency and will be considered in detail in a subsequent section concerned with blood lipid levels and coagulation. However, like the above tests, no clear pattern has emerged. Furthermore, these tests have usually been performed with diluted blood fractions and the results are again only a reflection of the concentrations of coagulation factors. For example, the prothrombin time has been estimated in ischaemic heart disease but in most instances where a shortening has been found, diluted plasma has been employed (Shapiro et al., 1942; McDonald and Edgill, 1957). This shortening is not apparent in tests with undiluted blood (Overman and Wright, 1951). The "dilute test" is, in fact, merely a measure of the amounts of Factor V, VII, X and fibrinogen present. Plasma fibrinogen is elevated after tissue injury and the shortened time with diluted blood after myocardial infarction for instance, reflects this elevation.

With undiluted blood, a shortening of the prothrombin time might be observed if certain clotting factors have already been activated *in vivo*, and such a shortening has been reported in venous thrombosis associated with visceral cancer (Ingram, 1954).

Ingram and Biggs (1953), although they did not comment upon the observation, show in Table II of their paper that the whole blood clotting time in silicone treated tubes decreases by approximately fifty per cent (20.4 seconds to 11.0 seconds) from the day of clinical onset of venous thrombosis to the second day after onset. Treating the tubes with silicone removes the contact effect with glass which otherwise occurs in the standard whole blood clotting time in untreated glass tubes. The above observation could be explained on the basis of activation having already occurred *in vivo*. Adelson and his colleagues (1961) claimed that when a massive thrombus is formed, there is a resultant hypercoagulable state which may be due to escape of thrombin from the thrombotic material into the systemic circulation. Under these circumstances a shortened clotting time in silicone treated tubes would be expected because the mechanism has already been activated.

The thromboelastograph is another method available for measuring variations in the speed of coagulation. An increased coagulability has been claimed in many cases of venous and arterial thrombosis, the changes being comparable with those

obtained experimentally immediately after the intravenous injection of thromboplastin (Matter, 1960). Nevertheless, the same objection exists as before, namely that the changes observed with the thromboelastograph may be the consequence of intravascular coagulation rather than indicating its cause, due to an inability to determine clinically the precise time of onset of thrombosis. That is, the technique may be indicating a hypercoagulable state induced by pre-existant thrombosis, in the manner suggested by Adelson et al. (1961).

Although there is no evidence that increasing the concentration of a clotting factor above what is assumed to be a normal level results in an increased tendency of the blood to clot, two procoagulant factors, namely fibrinogen and platelets, deserve further mention. An elevated fibrinogen level has been reported in pregnancy (Gram, 1922), in the postoperative period (Warren et al., 1950), post partum (Dieckmann and Wegner, 1934), in ischaemic heart disease (McDonald and Edgill, 1957, 1959; Katz et al., 1963; Eastham and Morgan, 1963) and in venous thrombosis (Brinkhous, 1948). An elevated platelet count has been recorded after operations (Dawbarn et al., 1928; Wright, 1942; Warren, 1953; Feruglio et al., 1960), post partum (Dawbarn et al., 1928; Wright, 1942) and in venous thrombosis (Gage, 1953). The concentration of circulating fibrinogen is one of the factors determining the viscosity of blood (Dintenfass, 1962; Merrill et al., 1963; Begg and

Hearns, 1966) and Dintenfass (1962) claims that he has demonstrated dramatic increases in the viscosity of blood after coronary thrombosis, at a time when the concentration of fibrinogen is elevated. An increased viscosity might lead to slowing in blood flow, with an increased likelihood of thrombosis. Although Begg and Hearns (1966) claimed that there was no demonstrable influence exerted upon the viscosity of blood by a considerable range in variation of plasma fibrinogen, and that the haematocrit value was of overwhelming importance in this regard, the effect of fibrinogen deserves further evaluation.

An increase in plasma fibrinogen might also affect platelet behaviour in view of the evidence, previously presented, that this substance appears to be a co-factor in ADP-induced platelet aggregation. Deykin et al. (1965) and Hardisty and Hutton (1966), however, have reported that with in vitro systems, maximal clumping activity was obtained at concentrations of fibrinogen of only 30 to 40 mg. per 100 ml., a level which is well below the normal range.

In addition to an increased platelet count, an increased platelet adhesiveness to glass was also reported by Wright (1942) in postoperative and post partum states, and by McDonald and Edgill (1957) in ischaemic heart disease. After splenectomy it seems that increased platelet adhesiveness alone does not alter the incidence of post-splenectomy thrombo-embolism, but when

combined with an elevated platelet count, increased adhesiveness may contribute to the pathogenesis of thrombo-embolic episodes. The elevated platelet count after splenectomy results in an increase in the total adhesive platelet count (Hirsh, McBride and Dacie, 1966). One might speculate that an increased platelet count may also contribute to thrombosis by increasing the total adhesive platelet count in ischaemic heart disease and other conditions in which there is a thrombotic tendency.

(ii) Decrease in the activity of coagulation inhibitors

The anticoagulant mechanism is an important factor in maintaining the fluidity of the blood, and Nossel (1964) states that it is "very likely that inhibitors occur to all of the intermediate products of coagulation". The dilution of the intermediate products in the presence of a rapid circulation of blood is probably an important ancillary to the inhibitors. The blood contains antithrombin activity (Astrup and Darling, 1942; Klein and Seegers, 1950), antithromboplastin activity (Tocantins, Carroll and Holburn, 1951) and an inhibitor of the contact product (Nossel, 1964), and it has been suggested that the anticoagulant system is controlled by a neuro-humoral mechanism (Kudrjashov, 1961).

It has been proposed that the blood might become hyper-coagulable due to a deficiency in these normal inhibitors, but apart from the rare examples cited earlier, this aspect has received little attention and the evidence that is available is of a

conflicting nature. The plasma of patients who have had a clinical thrombotic episode inactivates thrombin at the same or even at a greater rate than the plasma of normal individuals (Hurn, Barker and Mann, 1947; Paterson and McLachlin, 1954). Thrombosis has been reported, however, in association with the presence in plasma of excessive degrees of inhibitory activity against the fibrinolytic enzyme plasmin, and the suggestion was made that thrombosis occurs due to a failure to remove fibrin in the normal manner (Nilsson et al., 1961; Naeye, 1961). The death rate from coronary heart disease is significantly higher in the South African white population than in the Bantu, and assuming that thrombosis is an important factor in deaths due to coronary disease, the finding of Merskey and his colleagues (1960) that fibrinolytic activity is generally faster in the Bantu than in the white is of interest in view of the above observations. The latter workers, however, state that their observations might only be manifestations of hepatic disease in the Bantu. Obviously at the present, the evidence is fragmentary and the concept of impaired fibrinolysis leading to unrestricted deposition of fibrin and atherosclerosis needs further investigation. The subject of fibrinolysis will be pursued further in subsequent relevant sections.

Spaet and his co-workers (1961) think that the reticulo-endothelial system removes coagulant material from the circulation. According to their experimental work in rats

(Spaet and Kropatkin, 1958; Spaet, 1962), the reticulo-endothelial system and especially the liver, is a cellular clearing mechanism for the rapid removal of certain clotting intermediates from blood. The loss of this mechanism may be of importance in the formation of persistent thrombi. It has been reported that animals made hyperlipaemic are more susceptible than control animals to the formation of persistent thrombi after thrombin infusions (Greig and Runde, 1957; Teitlebaum, Cooperberg and Kalant, 1962), and perhaps this is due to the known influence of hyperlipidaemia on the reticulo-endothelial system (Kudrjashov et al., 1961).

Before proceeding to a discussion of lipids and blood coagulation, one further comment is pertinent to these remarks about laboratory investigations and the diagnosis of hypercoagulability. From the evidence presented it is unlikely, for various reasons already described, that a sample of a blood can be withdrawn from a patient and a diagnosis of hypercoagulability made. One more, and possibly very important reason for this inability, is that the changes occurring in the blood which predispose to thrombosis are probably only transient - Wessler's (1955) experiments support this remark.

The role of lipids in the coagulation system

Although lipids have long been known to be active in blood coagulation (Wooldridge, 1883), the exact nature of the lipid and its relationship to coagulation still remain uncertain despite intensive research stimulated by the possibility that dietary fat may play a role in thrombogenesis, In a review of the role of lipids in blood

coagulation, Merskey and Marcus (1963) stated that the lipid required for the formation of intrinsic thromboplastin is normally provided by platelets and that lipid derived from the plasma, the red cell membrane, and the white cells is probably unimportant. Lipids are also important in the extrinsic thromboplastic system (Chargaff, 1944).

There is considerable evidence that phosphatides are the most active class of lipids in blood coagulation (Chargaff, 1944; Marcus et al., 1962; Billimoria, Irani and Maclagan, 1965a) and particularly those derived from platelets (Sohar, Rosenthal and Adlersberg, 1957). At least five phospholipids have been demonstrated in platelets (Marcus, Ullman and Wolfman, 1960; Troup et al., 1960), including phosphatidylethanolamine and phosphatidylserine, both of which in an apparently purified state have been shown to be active in some coagulation tests (Merskey and Marcus, 1963).

Stearic acid is the principal saturated fatty acid in phosphatidylethanolamine and furthermore this phosphatide is predominantly in the plasmalogen form (Marcus et al., 1962), and although the biological functions of plasmalogens are not yet established, "it is possible that they have a role in the maintenance of cell membrane integrity" (Merskey and Marcus, 1963). An attempt to demonstrate whether plasmalogens are active in coagulation was inconclusive (Zilversmit, Marcus and Ullman, 1961).

Important questions, as yet unresolved, are the mechanism whereby platelet phospholipids participate in coagulation and where is the active lipid substance in platelets? Bounameaux (1957), and

Surgenor and Wallach (1961) have claimed that intact platelets are active in clotting systems; the latter suggested that the platelet membranes enter into coagulation by attracting a selected environment of clotting factors from the plasma, a view which is also shared by Marcus and his colleagues (1966) and Hardisty and Hutton (1966a). Recently Mustard and his colleagues (1964a) observed that the addition of ADP to native platelet-rich plasma causes an acceleration of clotting. Although platelets dramatically swell when exposed to ADP (Bull and Zucker, 1965), this is a reversible phenomenon and the platelets are obviously not disrupted by the process - perhaps the swelling with a resultant change in shape of the platelet makes the lipid in its membrane available for participation in clotting. In a recent ultrastructural study, White and Krivit (1966) demonstrated the transformation of platelet granules into particles with the configuration of lipid micelles, and the ejection of these micelles through intact cell membranes into the surrounding plasma prior to cell aggregation - perhaps the metabolic changes associated with platelet swelling initiate this process. It is of interest that thrombin also causes platelet swelling (Bull and Zucker, 1965). This, of course, is to be expected if the clumping activity of thrombin is mediated through ADP released from platelets (Kaser-Glanzmann and Luscher, 1962).

There are two possible ways in which the clotting process may be initiated - by the release of tissue thromboplastins into the circulation or by activation of the contact or Hageman factor.

The intravenous administration of tissue extract results in thrombus formation (Mason, 1924; Copley and Stefko, 1947) and excess tissue fluid entering the circulation after myocardial infarction may explain why this condition is often followed by venous and cardiac mural thrombosis. In a systematic study of the contact phase of blood coagulation, Nossel (1964) presented evidence that certain fatty acids including stearic acid activate the contact factor. On a speculative note, it is interesting to recall the significant content of stearic acid in platelet phosphatidylethanolamine and whether this might be the substance made "available" for activation of the Hageman factor when the shape of the platelet is altered, if indeed thrombocytic activation of clotting occurs as suggested by Iatridis et al. (1964).

Blood lipid levels and coagulation

The plasma contains much lipid and this is further elevated in some circumstances, for instance after a fat-containing meal. The question arises whether this lipid can be made available for coagulation and possibly promote thrombosis? It is of interest that the mean plasma lipid levels of individuals with clinical evidence of vascular disease are elevated when compared to apparently healthy subjects (Merskey et al., 1960). Because thrombosis may be important in the development of atherosclerosis (Rokitansky, 1852, Duguid, 1946) as well as complicating this condition, it is necessary to consider coagulation tests in "atherosclerotic subjects". Two lines of approach have therefore developed. Firstly, blood coagulation has been investigated in

patients suffering from "atherosclerotic disease", and secondly, such tests have been performed in patients with hyperlipidaemia, or in subjects in whom hyperlipidaemia has been induced.

The evidence for a thrombotic tendency in atherosclerotic heart disease (Subjects with clinical evidence of ischaemic heart disease), based on coagulation tests is quite inconclusive. The coagulation time was not accelerated in five studies (McDonald and Edgill, 1957; O'Brien, 1958; Merskey et al., 1960; Dailey et al., 1960; Schwartz et al., 1965), the Stypven time was accelerated in two (McDonald and Edgill, 1957; Billimoria et al., 1965b) and not in another study (O'Brien, 1958), the prothrombin time with other thromboplastins was normal in a further two studies (McDonald and Edgill, 1957; Merskey et al., 1960), and finally, thromboplastin generation was reported to be accelerated by one group of workers (McDonald and Edgill, 1957, 1959) and not by another group (Merskey et al., 1960).

Innumerable parameters of blood coagulation have also been assessed in response to lipaemia but again the results are conflicting. Indeed, Merskey and Marcus (1963) comment that "workers in the field may be broadly classified into those who believe that fat ingestion significantly affects blood coagulation, those who doubt this, and those who formerly believed this but now question the wisdom of such conclusions". There is almost complete unanimity of opinion, however, that the prothrombin time using Russell's viper venom is accelerated by lipaemia (Fullerton et al., 1953; Sohar et al., 1957;

O'Brien, 1958; Nitzberg et al., 1959; Schmidt and Clifford, 1960), Lewis (1960) failing to confirm this observation. The results of the Russell's viper venom time, however, are probably artefactual, because the test is particularly sensitive to the concentration of lipids in the plasma (O'Brien, 1960) and may therefore merely be a measure of an increase in plasma lipid. Thromboplastin generation has been reported to be increased (Schmidt and Clifford, 1960; Mustard and Murphy, 1962) and normal (Sohar et al., 1957; Nitzberg et al., 1959; Ollendorff et al., 1964; Boyles, 1965) and likewise the coagulation time using various techniques has been found to be either accelerated (Buzina and Keys, 1956; Keys et al., 1957; O'Brien, 1958; Mustard, 1958 Dailey et al., 1960; Slack et al., 1964) or normal in lipaemia (Tulloch et al., 1953; Manning and Walford, 1954; Sohar et al., 1957; Sheehy and Eichelberger, 1958; Borrero et al., 1958; Nitzberg et al., 1959; Lewis, 1960).

Moreover, there is no clear evidence that the type of dietary fat has any influence on coagulation tests. In six studies on subjects without clinical evidence of vascular disease the response was the same whether fats of animal or vegetable origin were used (O'Brien, 1956; Maclagan and Billimoria, 1956; Keys et al., 1957; McDonald and Fullerton, 1958; Farquhar et al., 1961; Ollendorff et al., 1964), whereas Mustard and Murphy (1962) reported that the prothrombin time and thromboplastin generation were accelerated by a diet high in egg yolk but were normal in response to a diet of dairy fat or a diet low in fat. In another study, subjects given a diet rich in

polyunsaturated fatty acids showed, after six months, a significant prolongation in the clotting time when compared with a similar number of subjects feeding on a normal diet (Buzina et al., 1961). When hyperlipidaemia was induced in subjects with clinically manifest vascular disease, the clotting time was shorter than in control subjects in one instance (Slack et al., 1964) but the same as in control subjects in two other studies (O'Brien, 1958; Dailey et al., 1960).

Today, the total serum cholesterol level is routinely performed in almost any general examination of an adult, and hypercholesterolaemia, moreso than any other elevated lipid fraction, has been investigated and incriminated in atherogenesis. Recently Friedman (1966) stated that it is statistically valid to consider that patients with an elevated cholesterol level are subject to a greater risk of developing vascular accidents than normocholesterolaemic subjects. This conclusion is based on the claim that an elevation of the total serum cholesterol is associated with the subsequent development of coronary heart disease (Kannel et al., 1964), that differences in the total serum cholesterol level exist in populations in which the prevalence of coronary heart disease varies (Shaper and Jones, 1959), that there are differences in the serum cholesterol level in subjects with and without myocardial infarction (Keys, 1951; Denborough and Nestel, 1964; Blacket et al., 1965), and a mass of other related observations. However, whether these correlations

imply a causal relationship is not yet definitely known.

It is apparent that no definite conclusions can be drawn as to the role of dietary fat in atherosclerosis on the basis of coagulation tests. This state of confusion is aptly reinforced by such studies as that of Goldrick and Whyte (1958) in which the coagulation time was found to be shorter in New Guinea natives, said to be free of atherosclerotic disease, than in white Australians. The conflicting nature of all these reports are due to many variables, including the crude nature of the tests used to assess coagulation, varying intervals of time of observations after fatty meals, diurnal variations in lipid status, difficulties in the diagnosis and assessment of severity of "atherosclerotic disease", and in the selection of control subjects.

Another approach has been to study the influence of dietary fat in the experimental animal, and the literature on this subject is extensive. Howard and Gresham (1964) recently reviewed this aspect. Hartroft and Thomas (1957) were able to produce myocardial infarction in rats with a combination of thioracil, cholesterol, sodium cholate and animal fat, and Gresham and Howard (1960) claimed that they could produce thrombosis and myocardial infarction if butter fat was fed to animals. In this latter study, it was additionally noted that if peanut oil rather than butter fat was administered, atherosclerosis was the predominant lesion. Taylor and his colleagues (1959) fed two Rhesus monkeys on a high fat diet for four years, the resulting serum cholesterol levels being respectively 554 and 659 mg. per 100 ml;

the first developed severe atherosclerosis of the arteries of the limbs and gangrene of the feet and the other died of myocardial infarction. The atherosclerotic lesions in the aortas of both monkeys were claimed to be similar to those found in man. Although the artificial nature of the diet in these studies makes it difficult to draw any analogy with human disease, the reports are of considerable interest and certainly warrant further investigation.

Numerous studies on blood coagulation in animals have been reported, but once again the findings have been discrepant, probably for the same reasons as in the human studies, but with the added problem of species differences. These studies have been mentioned in the review by Merskey and Marcus (1963) and cannot profitably be discussed here.

Epidemiologic investigations have constituted yet another approach to the role of dietary factors in thrombosis and occlusive vascular disease and this subject was recently reviewed by Bronte-Stewart (1965). As he points out, the interpretation of epidemiological data can be hazardous, and Yerushalmy and Hilleboe (1957) reinforced this view with respect to the significance of dietary fat in the genesis of atherosclerotic disease; the latter dramatically concluded that "the suggested association between national death rates from heart disease and percentage of fat in the diet available for consumption cannot at the present time be accepted as valid". The most significant factor in focussing attention on diet as a causative factor in occlusive vascular disease was probably the striking fall

in mortality from circulatory diseases during the second world war (Malmros, 1950; Strom and Jensen, 1951). Consistent with a dietary role, Thomas et al. (1960) and Gore et al. (1964 showed in parallel with the marked difference in racial mortality from ischaemic heart disease between Japan and the United States (Keys, 1957; Page et al., 1957; Loewenstein, 1964) a similar marked difference in the prevalence of thrombosis in the large veins of the leg at necropsy. On the other hand, communities reported to be consuming a high dietary intake of fat of animal origin, experience a low incidence of ischaemic heart disease (Hunter, 1960; Shaper, 1962; Mann et al., 1964). In retrospective studies, Blacket and his colleagues (1965) claimed that patients with myocardial infarction had taken a diet significantly higher in fat than did control groups, but other investigators (Little et al., 1965; Paul et al., 1963; Papp et al., 1965) were unable to detect any difference in diatary fat content between patients with and without ischaemic heart disease. A multifactorial basis for atherosclerosis and thrombosis, and this is probably so, could account for these discrepant observations, and the role of dietary fat cannot therefore be discounted.

These retrospective studies are always suspect due to limitations in the accuracy of assessment of the diet; the argument might be resolved at least in part, if it were shown that restriction or modification of dietary fat intake reduced or modified the incidence of thrombotic disease and atherosclerosis. Several prospective therapeutic trials have been performed, but when

drawing any conclusions from such studies, it must be remembered that atherosclerosis is probably a lifelong process (Strong and McGill, 1963). All studies to date have been with elderly subjects - ideally the trials should be commenced in childhood. Hansen and his colleagues (1962) studied a large number of subjects in a geriatric hospital, all over the age of 60 years, and claimed to have obtained significant evidence that a diet rich in vegetable oils reduced the incidence of clinical thrombosis. Lyon et al. (1956), Morrison (1960) and Bierenbaum et al. (1965) reported the effects of dietary trials in survivors of myocardial infarction and claimed a significant benefit in treated subjects. The Anti-Coronary Club Project in New York (Christakis et al., 1966) has recently reported data indicating a favourable effect from a serum cholesterol-lowering diet in males aged 40-59 years and free of coronary heart disease. In the United Kingdom a research committee under the chairmanship of K. P. Ball (1965), however, found no difference in the recurrence rate or death rate in survivors of myocardial infarction whose dietary fat was limited to 40 grammes daily, compared with survivors whose intake of fat was not modified.

Although these various studies can all be criticised for one or more reasons, the trials have mostly shown a benefit, and further trials are desirable. On the Australian scene, Fairbairn and Whyte (1965) presented data on coronary deaths and serum cholesterol levels in Australian males. The composition of the diet was discussed and they speculated on the possible effects of

altering the diet, so as to decrease the serum cholesterol level, on the assumption that a dietary induced alteration in cholesterol level may be accompanied by a reduction in coronary disease.

In 1962, Oliver reported that a mixture of androsterone and clofibrate (atromid) lowers the blood cholesterol and triglycerides. In the following year he found that clofibrate without androsterone (atromid-S) appeared to have the same effect (Oliver, 1963). Both compounds produced a statistically significant and similar fall in serum cholesterol in 35 men with ischaemic heart disease (Carson et al., 1966). In view of the alleged relationship between hypercholesterolaemia and coronary heart disease, these observations have provoked much interest and accordingly these compounds are being widely evaluated at the moment.

Fatty acids, coagulation and thrombosis

The observation that fatty acids promote blood coagulation is not a recent one, Stuber and Heim apparently having first described this phenomenon in 1916. The current studies of this problem were initiated by Poole in 1955, who showed that certain fatty acids, in low concentration, accelerate the coagulation time of recalcified citrated human plasma. Others have confirmed these findings (Pilkington, 1957; O'Brien, 1957). Connor, Hoak and Warner (1965) recently demonstrated that long chain saturated fatty acids shorten the clotting time of human whole blood and native plasma in silicone-coated glass tubes, no anticoagulant being employed in their system.

In 1961 Connor and Poole extended these observations by exploring the effects of the sodium salts of various fatty acids on the formation of artificial thrombi in the Chandler apparatus. Straight chain, saturated fatty acids with between sixteen to twenty six carbon atoms greatly accelerated the thrombus formation time of rat blood and longer thrombi were produced. The shorter thrombi did not appear to result from a lesser fibrin content and they suggested that "the conversion of fibrinogen into fibrin had somehow been altered so that a loose, more strung-out configuration resulted". Short chain saturated and long chain unsaturated fatty acids were inactive in this regard. The effect of sodium stearate was abolished after two hours incubation with five percent bovine albumin. This finding is discordant with the earlier claims of Poole (1955) that stearate suspended in an albumin solution promoted clotting just as effectively as when suspended in distilled water.

The effects of the fatty acids have been attributed to the activation of the Hageman factor by Connor (1962), Margolis (1962) and Nossel (1964). The activity of the sodium salts of the saturated fatty acids might be due to the particulate nature of these acids in aqueous solutions (Jirgensons, 1958), and it is of interest that glass, kaolin and bentonite acted in vitro like long chain, saturated fatty acids (Connor, 1962).

The administration of long chain saturated fatty acids in vivo also produces thrombosis. The injection of unbound, long chain, saturated fatty acids into the systemic circulation of dogs produced

a massive generalised thrombosis and death (Connor, Hoak and Warner, 1963). With blood taken just before death it was shown that the whole blood clotting time in silicone-coated tubes was markedly reduced. Long chain unsaturated fatty acids were not lethal but did produce thrombi in isolated jugular vein segments. Hoak, Connor and Warner (1966) subsequently claimed that albumin-bound stearic and oleic acid were also thrombogenic but the concentrations of fatty acids were so high that it is not unlikely that some unbound fatty acid was also present in the solutions injected. In fact, in an earlier study (Hoak et al., 1964) the same workers showed that generalised thrombosis did not occur when stearic acid was incubated with albumin. These discrepancies can be attributed to variations in the amount of fatty acid incubated with albumin and to variations in the length of time of incubation of fatty acid with the albumin prior to injection.

Soloff and Wiedeman (1963) injected 0.01 percent suspensions of the sodium salts of stearic and palmitic acid into the major artery of the unanaesthetized bat's wing and confirmed the in vivo thrombogenic effects of these two fatty acids. Oleic and linolenic acid were without effect.

The non esterified fatty acids are carried in the blood bound to albumin (D. S. Goodman, 1958), and in this form their properties are probably modified (Gordon and Cherkes, 1956). Although the evidence which has been presented is conflicting, it seems likely that the binding of free fatty acids to albumin will inhibit or reduce

their thrombogenic activity. What, therefore, is the relevance of the foregoing observations to clinical disease in man? Hoak and his colleagues (1964) raise the theoretical possibility that a transient metabolic change might occur with a portion of the plasma fatty acids becoming temporarily unbound or loosely bound. The concentration of free fatty acids in the plasma increases significantly following the injection of ACTH and other pituitary extracts due to a mobilisation of depot fat (Raben and Hollenberg, 1958; Di Girolamo et al., 1961). This prompted Hoak, Poole and Robinson (1963) to explore the possibility that these substances might cause thrombosis. Subcutaneous injections of ACTH or porcine anterior pituitary extract in normal rabbits produced a five to seven fold rise in plasma free fatty acids and thrombi were found in the lungs in seven of ten rabbits. This study therefore suggests that a disturbance in the normal binding mechanism may occur.

There are several conditions in man in which thromboembolic complications occur and in which elevated levels of free fatty acids have been reported. "Stress", probably due to a release of epinephrine, results in a rise in plasma free fatty acids, and there are innumerable reports that emotional stress may be a factor in the genesis of myocardial infarction in man (Weiss et al., 1957; Friedman and Rosenman, 1959; Pearson and Joseph, 1963; Russek, 1965; Minc, 1966). The plasma free fatty acid level tends to be higher in patients with ischaemic heart disease than in apparently healthy persons (Schrade, Biegler and Bohle, 1961). The

Framingham and Albany studies have suggested that cigarette smoking may be an important factor in the development of coronary heart disease (Doyle et al., 1962) and it is not without interest that a marked rise in free fatty acid levels occurs after smoking cigarettes (Kershbaum et al., 1961). In patients with diabetes mellitus, the free fatty acid level is elevated (Bierman et al., 1957), and Beckett and Lewis (1960) have reported that thromboembolic complications are prominent in obese diabetic patients placed on rigid weight reduction diets. They suggest that the excessive mobilisation of body fat may be an aetiological factor in occlusive vascular disease in diabetes mellitus. Finally, it has been claimed that there is a tendency to develop thrombosis in women taking steroid oral contraceptives (Minogue et al., 1963), and it has been shown that the same steroid drugs significantly lower the level of plasma albumin (Pilgeram and Pickart, 1963). Thrombosis might conceivably develop in this circumstance due to ineffective binding of plasma fatty acids.

In the light of the experimental evidence presented, the various clinical conditions cited suggest that further investigation into the role of elevated levels of plasma free fatty acids in the development of thrombosis in man is warranted. However, there is no conclusive evidence at present that such a mechanism is important.

Fibrinolysis

It is not inconceivable that thrombosis may be mediated through mechanisms which interfere with thrombus dissolution

rather than by mechanisms which promote coagulation. Two reports (Nilsson et al., 1961; Naeye, 1961) mentioned earlier in this review, support this suggestion; excessive degrees of inhibitory activity against the fibrinolytic enzyme plasmin were demonstrated in several patients with thrombosis.

Sherry, Fletcher and Alkjaersig (1959) in a review of the fibrinolytic system in man, defined the term "thrombolysis" as the phenomenon by which a thrombus is lysed by virtue of activity of the plasminogen system. The knowledge that plasmin would digest fibrin led earlier investigators to assume that under certain circumstances plasminogen is activated in the body and plasmin thus formed digests fibrin. This classical view of lysis by hyperplasminemia, which occurs when the rate of activation of plasminogen exceeds the inhibitory activity of the blood, does not, however, explain the known facts in fibrinolysis. The observation that plasminogen and activator are both absorbed onto fibrin during the process of clotting (Sherry, 1954; Sherry and Alkjaersig, 1957) prompted an alternative explanation for the phenomenon of fibrinolysis (Alkjaersig, Fletcher and Sherry, 1959). According to this view, thrombolysis results from absorption of plasminogen activator onto a thrombus with resultant activation of the intrinsic plasminogen of the thrombus. This concept allows for thrombolysis without evidence of generalised proteolysis in the circulation, and moreover, this mechanism can operate relatively independently of the presence of circulating plasmin inhibitors; the classical hypothesis could not explain either of these observations.

With the recognition that fibrinolysis could be ascribed to the activation of plasminogen on the fibrin surface, attention was then directed to the nature and source of the plasminogen activator. Its origin in the body is still uncertain, however. Hedin (1903) and Nolf (1908) supposed that fibrinolysis was brought about by an enzyme released from degenerating leucocytes. Nolf (1908) also considered that an enzyme may be derived from endothelial cells, as did Mole (1948). The most direct evidence that the vascular endothelium is the source of the activator has come from Todd (1958, 1959), who incubated frozen sections of fresh tissues on thin films of fibrin and demonstrated zones of fibrinolysis clearly related to endothelium, especially venous endothelium. On several occasions, Warren (1963, 1964, 1965) has also demonstrated that, of the components of the vessel wall, the endothelial layer is unique in that it alone possesses the ability to induce lysis of fibrin. He concluded (1964) that endothelium released plasminogen activator which diffuses into thrombi, with the conversion of plasminogen to plasmin which is in close contact with the fibrils of fibrin.

The hypothesis proposed by Alkjaersig et al. (1959) has been tested and confirmed experimentally (Soardi et al., 1960) on thrombi produced in the manner of Wessler (1955). Recently, however, Hedner et al. (1966) claimed that fresh human thrombi obtained post mortem contained, at the most, only traces of plasminogen and therefore concluded that "intrinsic clot lysis" may not be the main mechanism of thrombus dissolution. Moreover,

platelets contain a fibrinolytic inhibitor (Alkjaersig, 1961), and the high platelet content of many thrombi, especially arterial thrombi, could significantly inhibit the activation of plasminogen. With *in vitro* thrombi produced in a cone-in-cone rotational viscometer, streptokinase induced lysis of fibrin but platelet masses remained unaffected (Rozenberg, 1965). The failure of intravenous "thrombolysin", a fibrinolytic agent, in the treatment of acute coronary thrombosis could also be related to the prominent platelet component of coronary artery thrombi (Dewar *et al.*, 1963).

Effect of plasma lipids on fibrinolysis

There has been a lot of interest in this subject, principally to assess whether there is any relationship between plasma lipids, impaired fibrinolytic activity and myocardial ischaemia. A review was published by Howell in 1964. The problem has been approached in many ways.

A fat-containing meal has been reported to inhibit fibrinolysis (Greig, 1956; Billimoria *et al.*, 1959; Farquhar *et al.*, 1961) but this has been denied by other workers (Houghie and Ayers, 1960; Kowarzyk *et al.*, 1960). Gajewski (1961) claimed that inhibition occurs only in subjects with ischaemic heart disease, and not in normal subjects. In an addendum to his paper, Greig (1956) stated that further work had shown that the lipaemia resulting from the ingestion of unsaturated fats as opposed to a diet rich in saturated fats, did not inhibit fibrinolysis.

The above experiments, according to Howell (1964), are

assessing the effect of chylomicrons upon fibrinolytic activity; she, however, suggests that epidemiological surveys indicate that B-lipoproteins are "most important in the aetiology of myocardial ischaemia". In comparisons between Europeans and the Bantu (Merskey, Gordon and Lackner, 1960) and with New Guinea natives (Goldrick, 1961), the Europeans were shown to have less fibrinolytic activity and higher plasma levels of B-lipoprotein. Because of other differences, however, it is not possible to conclude whether there is any causal relationship between these two parameters.

Numerous experiments in animals have also confirmed the inhibitory effect of lipids on fibrinolytic activity. Cholesterol feeding in rabbits inhibits fibrinolysis in vivo (Kwaan and McFadzean, 1957) and other studies in which inhibition was noted when hyperlipidaemia was produced with a diet rich in saturated fats are those of Bang and Cliffton (1960) and Furuta (1962). These three in vivo studies certainly suggest that lipids do inhibit fibrinolysis, but the mechanism remains unexplained.

An analysis of fibrinolytic activity in patients with coronary heart disease as compared to normal subjects reveals no demonstrable alteration in most studies (Merskey et al., 1960; Nestel, 1960). Ogston and Fullerton (1965) demonstrated a lowered plasma fibrinolytic activity sixteen-forty eight hours after myocardial infarction and suggested that this low activity might represent a continuation of a low level of fibrinolysis present before infarction, and which played a part in the causation

of coronary thrombosis. At present there is no conclusive evidence of impaired fibrinolytic activity in patients with ischaemic heart disease.

The concept of a dynamic haemostatic balance in the organism between continuous fibrin formation and fibrin dissolution has been in existance since the beginning of this century and has been championed in recent years by Astrup (1964). He has proposed that a disturbance in this dynamic process is the basis of the thrombogenic theory of atherosclerosis. Furthermore, he has stated that the effects of lipids can easily be incorporated into the structure of the thrombogenic theory - lipids could be involved in the process in many ways and it is of interest that in the list an inhibitory effect of lipids on the activity of the fibrinolytic system has been included.

Diabetes mellitus and occlusive vascular disease

Evidence that occlusive arterial disease is even more common in diabetic than non-diabetic individuals has been well documented in several clinical studies (Liebow et al., 1955; Marble, 1955; Bryfrogle et al., 1957). Moreover, in these studies the arterial complications were reported to become manifest at an earlier age in the diabetic subjects, although there are dissentient views (Pathania and Sachar, 1961). Sievers and colleagues (1961) showed that the prevalence of clinical diabetes mellitus was some five times greater in a large series of patients with myocardial infarction than in the general Swedish population.

These clinical impressions have been extended in more recent investigations. In the Bedford diabetes survey (Keen et al., 1965) a correlation between elevated blood sugar levels and symptomatic or electrocardiographic evidence of arterial disease has been demonstrated. Ostrander and associates (1965) have shown, in a most interesting epidemiological survey of the community of Tecumseh, Michigan, U.S.A., that the frequency of elevated blood sugar levels is significantly greater in patients with coronary, cerebral or peripheral vascular disease. The latter suggested that the most obvious explanation of this association is that "many individuals with vascular disease are mild or latent diabetics" in whom the vascular manifestations overshadow the carbohydrate abnormality.

In recent years, the concept of a preclinical diabetic state has emerged, and its relationship to the development of coronary artery disease was recently reviewed (Herman and Gorlin, 1965). It was noted that among patients with premature coronary artery disease, an abnormally high number have preclinical diabetes. This observation is consistent with the conclusions of Ostrander et al. (1965) and Wahlberg's (1962) finding of abnormal intravenous glucose tolerance tests in forty six percent of non-diabetic subjects with vascular disease, as compared with only ten percent in control subjects without clinical evidence of occlusive vascular disease. Sowton (1962), Kimber and Phear (1965) and Reaven et al. (1963) have all reported abnormal glucose tolerance tests, consistent with preclinical or clinical diabetes mellitus, in subjects after myocardial

infarction.

Liebow and colleagues (1955) in their study of 383 diabetic out-patients found manifestations of arteriosclerotic heart disease (New York Heart Association Criteria) in forty two percent of patients; the prevalence of heart disease showed no relationship to the total serum cholesterol level, the degree of diabetic control, body weight, the daily insulin dose, or the duration of the diabetes. Whether the relationship reflects an enhanced atherogenesis, an increased thrombotic tendency, or both, in diabetes mellitus, or whether both diabetes mellitus and vascular disease (atherosclerosis and/or thrombosis) have a common genesis is not known. In an attempt to elucidate the mechanisms for this impaired glucose tolerance, Vallance-Owen and Ashton (1963) found that nineteen out of twenty eight unselected patients with myocardial infarction compared with six out of the same number of matched controls, had increased antagonism to insulin associated with their plasma albumin - an abnormality found in clinical and preclinical diabetes mellitus. Elevated plasma-insulin levels have been demonstrated after myocardial infarction (Peters and Hales, 1965; Nikkila et al., 1965) and in patients with peripheral vascular disease (Welborn et al., 1966).

Dietary carbohydrate and ischaemic heart disease

A possible relationship between dietary fat and coronary heart disease has already been discussed. This hypothesis has received a vast amount of attention, but in a cautionary note on

the interpretation of, and conclusions drawn from epidemiologic studies related to this subject, Yerushalmy and Hilleboe (1957) concluded that the suggested association cannot yet be accepted as valid, and in fact found heart disease to be more closely associated with animal protein in the diet.

To complete the triad of major dietary components, there has, in the present decade, been some speculation about the part played by refined carbohydrates, mainly sucrose, in causing ischaemic heart disease. Yudkin (1964) has reviewed the epidemiological data available on carbohydrate in the diet, and finds that the national level of consumption of sugar and fat are closely similar in twenty two countries. He concludes that "statistics relating fat intake to ischaemic heart disease or diabetes mellitus in different populations may therefore express only as indirect relationship, and the causal relationship may be with sugar". In a retrospective study, Yudkin and Roddy (1964) showed that subjects with peripheral arterial disease, or with a recent first myocardial infarction, consumed significantly more sucrose than control subjects. Papp et al. (1965) and Little et al. (1965), however, did not confirm this difference, but the subjects included in each study are not comparable.

In an interesting review of carbohydrate metabolism and cardiovascular disease, Albrink (1965) has traced the alteration in man's diet, from one composed chiefly of protein and fat changing to one largely of carbohydrate occurring with the advent

of agriculture 10,000 years ago. In more recent years there has been an increased availability of highly purified carbohydrate with an increased intake of total calories of all kinds. She concluded that diabetes mellitus and atherosclerosis may be "manifestations of the effect of affluence on a once useful genetic trait, the ability to conserve carbohydrate".

Plasma triglyceride levels are increased in ischaemic heart disease (Denborough and Nestel, 1964) and it has been reported that triglyceride concentrations show a closer correlation with coronary artery disease than does the serum cholesterol level (Albrink et al., 1961). It is, therefore, not without interest that in normal men an increase in plasma triglyceride concentration and changes in fatty acid composition accompany the consumption of a diet rich in carbohydrates, whereas a diet rich in animal fat produced no demonstrable changes in triglyceride metabolism (Nestel and Hirsh, 1965). Subsequently, Nestel (1966) demonstrated that subjects with coronary heart disease show a similar response to consumption of excess carbohydrate. He concluded that in both normal subjects and in those with coronary heart disease, the hypertriglyceridaemia was due to both increased production and decreased removal, and that "carbohydrate is a major determinant of plasma triglyceride turnover". Prompted by Yudkin's (1964) suggestion that sucrose may have an aetiological role in ischaemic heart disease, Rifkind and his associates (1966) investigated and found that short-term

sucrose restriction produced an initial significant fall in serum triglyceride level.

Lipid metabolism in diabetes mellitus

Serum triglycerides, not unexpectedly, may be elevated in diabetes mellitus (Albrink and Man, 1958). Schrade and his colleagues (1963) investigated in detail the fatty acid composition of lipid fractions in diabetic serum; the results depended somewhat on the state of control of the diabetes. Quantitative and qualitative abnormalities involving a wide range of lipid fractions were noted. Bierman and his associates (1957) had earlier observed abnormalities in non-esterified fatty acid metabolism in diabetes mellitus. The well documented alterations in lipid metabolism in diabetes mellitus may relate to the increased incidence of arterial disease in diabetes mellitus in view of the claims amassed for an association between lipids, blood coagulation, fibrinolysis and thrombosis.

Fibrinolytic activity in diabetes mellitus.

In two studies (Hathorn et al., 1961; Fearnley et al., 1963) fibrinolytic activity was lower in diabetic subjects, but in a third study (MacKay and Hume, 1964) no difference was noted when compared with control subjects. Hathorn and his co-workers (1961) claimed that Indian diabetics in Natal are far more prone to overt forms of vascular disease than Zulus with diabetes living in the same country; plasma fibrinolytic activity was lower in the Indian diabetics and there was a significant association between depressed fibrinolytic activity and high plasma-lipid levels in these subjects.

The claim that fibrinolytic activity is decreased in diabetes mellitus is an interesting observation, but requires further investigation before it can be implicated as a link between abnormal carbohydrate metabolism and the development of arterial disease.

A qualitative abnormality in platelets predisposing to thrombosis

So far the discussion has been confined to an analysis of abnormalities in the coagulation mechanism as a possible explanation for a generalised change in the circulating blood predisposing to thrombosis. Although platelet behaviour, and in particular platelet aggregation, is intimately associated with the coagulation mechanism, the question has recently been raised whether the generalised change in the blood, if such exists, is due to a primary change in the platelet? Platelets have an important role in thrombosis, and perhaps an increased platelet "stickiness" unrelated to the coagulation sequence may be the prime factor in thrombogenesis.

In a review of the role of blood platelets in thrombo-embolism, Moolten and his colleagues (1949a) rather prematurely concluded that "evidence has now accumulated in ample measure that a relation exists between the increased stickiness of platelets and an abnormal susceptibility to thrombus formation". They stated that one of the first reports of an increase in "platelet agglutinability" was in 1928 - in patients recovering from surgical procedures, and went on to discuss the various techniques for assessing platelet stickiness which have been employed since that time. At present the evidence

is, in fact, too scanty for adequate assessment. Nevertheless, platelet stickiness or adhesion to glass as determined by the method of Wright (1941), has been reported to be increased in patients with ischaemic heart disease (McDonald and Edgill, 1957, 1959; Slack et al., 1964). McDonald and Edgill (1959) claimed that the values for platelet stickiness significantly increased fromhealthy controls to patients with angina pectoris, and from these to patients with cardiac infarction. In another study, McDonald and Edgill (1958) claimed a significant decrease in platelet stickiness in patients with ischaemic heart disease maintained on a low fat diet for four to five weeks. Employing a turbidimetric technique (Born, 1962; O'Brien, 1962b), Rozenberg and Firkin (1966), however, found platelet aggregation in the presence of ADP and thrombin to be within the normal range in patients with arterial or venous thrombosis. These discordant views may be due to the different techniques employed - the Wright (1941) technique probably measures both platelet adhesion to glass and platelet aggregation.

Employing yet another technique, the glass-wool filter method of Moolten et al. (1949b), Murphy and Mustard (1962) found that the mean platelet adhesive index was significantly greater in subjects with occlusive vascular disease than in normal healthy controls. On the assumption that the ability of platelets to interact with each other and with surfaces, are of importance in determining platelet survival and turnover, these investigators also determined platelet survival with DFP^{32} in these subjects. Mean platelet survival was

shorter and mean platelet turnover was greater in the subjects with vascular disease. Furthermore, division of the subjects according to family history of "atherosclerosis" proved interesting. The most clearly separated groups were the subjects with both occlusive vascular disease and a positive family history and the controls with a negative history. Platelet survival can, however, be prolonged in human subjects by giving heparin and may be longer than average in human subjects with deficiencies in Factor VIII or Factor IX (Mustard, Rowsell and Murphy, 1966). This raises once again the question whether the altered platelet behaviour is secondary to changes in the coagulation mechanism?

There is evidence that environmental and other factors associated with the evolution of thrombosis and vascular disease influence the platelet and platelet survival and turnover in man and experimental animals, and these will now be discussed.

Lipids, platelet aggregation and thrombosis

Haslam (1964), Kerr et al. (1965) and Mahadevan et al. (1966) have all recently shown that long chain saturated fatty acids induce platelet aggregation in vitro. Haslam has suggested that this aggregation is mediated by the release of ADP from the platelet. Whether this release is related to the activation of Hageman factor, a property of fatty acids established by Nossel (1964), is not known. The particulate nature of the less soluble saturated fatty acids (Jirgensons, 1958) may evoke a phagocytic response from the

platelet with consequent nucleotide release and platelet aggregation in the manner suggested by Movat et al. (1965) for latex particles.

The ability of saturated fatty acids to induce platelet aggregation, rather than any effect on coagulation may be the explanation for the ability of these substances to produce thrombosis when injected into animals (Connor, Hoak and Warner, 1963).

In the last three years there have been several studies related to the effects of lipids and platelet aggregation in vivo. The extracorporeal shunt system provides a method by which this problem can be explored using dietary changes which are less dramatic than some of those employed in animal feeding alone, and moreover, a quantitative assessment of thrombosis is possible. Mustard and his colleagues (1963), using pigs fed on several different diets found that the weight of thrombus formed in the shunt was higher in pigs receiving added fat in their diet than control groups. An egg-yolk diet produced the highest mean weight of deposit, being more than three times as great as the deposit in animals given a lard-enriched diet. In general, it seemed that the saturated fats had a greater effect than the unsaturated fats. It was claimed that there was a significant correlation between blood cholesterol level and weight of deposit. In this experiment, the platelet adhesive index was also higher in the pigs receiving added fat in their diet, but none of the coagulation tests showing any significant differences from control animals. It is tempting to suggest, as did Mustard and his associates (1964), that one means by which lipids could influence the development

of at

of atherosclerosis is by increasing the amount of deposit which forms on the surface of the vessels.

Born and Philp (1965) studied the effects of lipaemia in rats, induced by feeding 5 ml. of butter administered by a stomach tube, on platelet thrombi which formed in the mechanically injured veins of the cerebral cortex (a technique first described by Florey in 1925) and found that the rate and duration of "white body" formation increased. Moreover, large white bodies formed in veins which had accidently been traumatized slightly by the experimental procedure - this was never seen in non-lipaemic rats. Although adenosine or large doses of heparin alone each inhibited white body formation in normal rats, both had to be given simultaneously in lipaemic animals to inhibit formation.

In two studies based on the incidence of thrombosis in pulmonary vessels in rats sacrificed three minutes after the injection of ADP, it was demonstrated that a diet containing saturated fat and cholesterol (Nordoy and Chandler, 1964) and intravenously administered cephalin, which contains a mixture of phospholipids (Nordoy and Chandler, 1965) both significantly increased the incidence of ADP-induced thrombosis. In the first study the diet of saturated fat alone did not induce thrombosis, and a diet of unsaturated fat (linseed oil) did not significantly affect the incidence of thrombosis. One possible interpretation of these various in vivo studies is that hyperlipidaemia increases the adhesiveness of platelets. ADP-induced platelet adhesiveness, using a modified Hellem (1960) technique, has been tested on platelet-rich plasma

from rats on a normal, on a saturated fat and cholesterol diet, and on this last diet supplemented with corn oil or linseed oil. An increased platelet adhesiveness was found in animals given a saturated fat and cholesterol diet. Corn oil in the diet further increased the adhesiveness, whereas linseed oil resulted in normal platelet adhesiveness (Nordoy, 1965).

In 1964, Owren and his colleagues suggested that thrombosis may be due to an increased tendency to platelet aggregation due to a deficiency of linolenic acid in the diet. This was not the first time that a dietary deficiency had been implicated in atherosclerosis and related phenomena (Sinclair, 1956). This hypothesis, and the grounds for such a suggestion were subsequently discussed in detail by Owren (1965). However, later in the same year Owren and his colleagues (1965) stated that they had not been able to confirm earlier reports that linolenic acid reduced platelet adhesiveness. A prophylactic trial for a period from three to eight months of linolenic acid in patients with coronary heart disease, conducted by Borchgrevink and his associates (1965) showed no difference in two groups, one given corn oil (1% linolenic acid) and the other linseed oil (50% linolenic acid). In two other studies, however, linolenic acid seemed to have a beneficial effect on platelet adhesiveness. In an experiment cited earlier (Nordoy, 1965), a diet containing linseed oil normalized platelet adhesiveness in rats feeding on a diet of saturated fat and cholesterol. Kerr

et al. (1965) claimed that linolenate inhibited platelet aggregation induced in vitro by saturated fatty acids. Further investigation will be necessary to evaluate the role, if any, of linolenic acid in thrombosis.

The effect of clofibrate with, and without, androsterone, on serum cholesterol levels was mentioned earlier (Carson et al., 1966) and these compounds are being subjected to investigation at the present time. In the same study, Carson and colleagues also claimed that both drugs favourably decreased platelet stickiness in patients with ischaemic heart disease, clofibrate and androsterone (atromid) being more effective than clofibrate alone (atromid-S). Gilbert and Mustard (1963) described similar effects of atromid on platelet "economy", and other investigators have also confirmed this observation.

Adrenaline, platelet aggregation and thrombosis, and stress

In 1914 Cannon and Gray showed that small doses of adrenaline promoted coagulation in vivo, and that larger doses inhibited it. Now, half a century later, several workers (O'Brien 1963b; Mitchell and Sharp, 1964) have demonstrated that adrenaline, and nor-adrenaline, also promote platelet aggregation in vitro. Further evidence linking adrenaline with the platelet has been presented. Platelet survival is shortened and platelet turnover increases (Adelson et al., 1961; Ozge and Mustard, 1964), and the platelet count in the peripheral blood is increased (McClure et al., 1965; Ozge et al., 1966). Accompanying the increase in platelet count there was also an

increase in platelet adhesiveness as measured by the Hellem (1960) technique (McClure et al., 1965); the observations were made by infusing adrenaline into young healthy males, and pronethalol, a β-blocker and phentolamine, an α-blocker, both prevented these changes. Employing normal and Factor IX deficient dogs, Ozge et al. (1966) concluded that the accelerating affect of adrenaline on clotting is not mediated through increase in activity of a specific clotting factor - perhaps the effect is mediated through an interaction between adrenaline and platelets.

Rowsell and his colleagues (1966) have confirmed the biphasic clot promoting effect of adrenaline and its ability to increase the circulating platelet count. More significant perhaps is the additional observation that adrenaline also quantitatively promotes thrombus formation (again biphasically) in an extra-corporeal circulation. The problem is far from resolved and several possibilities can be proposed to explain the thrombogenic properties of adrenaline. Haemodynamic effects seem unimportant, because over a wide range, flow bears little relationship to the weight of the deposit (Downie et al., 1963). One has to consider the effects of adrenaline on plasma free fatty acids (Shafrir and Steinberg, 1960), because of the known effects of fatty acids on platelet aggregation and thrombosis. Shimamoto et al. (1959) have claimed, from a study of the micro-circulation in the rabbit, that adrenaline enhances the adherence of platelets to the endothelium and that the morphology of the endothelial cells is altered. A possible conclusion from this study is that the

effect of adrenaline on thrombosis is mediated through damage to the endothelium. Shimamoto and Ishioka (1962) have also described an oedematous reaction in the intima of the arterial wall and suggested that there is an associated release of thromboplastic material. Two objections to this conclusion arise. Firstly, how does this account for the increased thrombus formation in the silicone-coated plastic of the extra-corporeal shunt and secondly, Hughes and Tonks (1962) have presented evidence that the vessel wall damage may, in fact, be secondary to the presence of platelet clumps on the vessel wall.

This leaves one explanation, namely, that the effects are due to a primary interaction of adrenaline with the platelet. O'Brien has described other properties of the adrenaline-platelet response which may well be relevant to human thrombosis. In ten apparently healthy subjects of both sexes, he demonstrated a marked variation in response from day to day aggregation being almost absent on some days. No cause for this variability could be detected. He found that the only way sensitive platelets could be made resistant was by incubating them with adrenaline (O'Brien, 1964a). This observation may be significant if adrenaline proves to have an important role in thrombosis. Secondly, he showed (O'Brien, 1963b) that phentolamine, a specific inhibitor of adrenaline, caused prolongation of the bleeding time. This prompts one to speculate whether adrenaline may normally be involved in platelet adhesion and aggregation in the haemostatic process, and therefore possibly in the development of thrombosis.

The findings in these experiments with adrenaline are also of some interest in view of the wide speculation about the relationship between emotional stress and the development of vascular disease and its complications (Weiss et al., 1957; Friedman and Rosenman, 1959; Pearson and Joseph, 1963; Minc, 1966). Although an association is implicated in all of these, and many other studies, the definition of "stress" varies widely. Friedman and Rosenman (1959) described a group of 83 men who manifested "an intense, sustained drive for achievement" and who were continually being "involved in competition and deadlines, both at work and in their avocations". The incidence of coronary artery disease was high in this group; the serum cholesterol level was also elevated. They labelled this behaviour pattern as type A. J. C. Paterson (personal communication) has aptly described this type of person as manifesting a "Sisyphus complex". Adrenaline, due to a direct effect on the platelet or the coagulation mechanism, or perhaps due to its ability to release plasma free fatty acids (Shafrir and Steinberg, 1960) may be the link between emotional stress and vascular disease. Paterson (1954) raises the additional point that transient elevations in blood pressure may occur with stress and cause rupture of capillaries in atherosclerotic plaques. He has long championed the view that intramural haemorrhage is an important factor in the genesis of atherosclerotic plaques and the development of thrombosis in association with these plaques.

Smoking and Thrombosis

Epidemiological evidence for an increased incidence of coronary artery disease has been claimed in several reports (Doll and Hill, 1956; Hammond and Horn, 1958; Bronte-Stewart, 1961; Doyle et al., 1962; Mulcahy and Hickey, 1966). There is, however, evidence to the contrary (Haag and Hanmer, 1957), and in a survey of 12,000 men in fourteen occupational categories, Russek (1965) concluded that his observations casted doubt upon the alleged role of smoking in the genesis of coronary heart disease and added further emphasis to the probable influence of emotional factors in its causation.

Several changes occur following the smoking of cigarettes. Free fatty acids are mobilised (Kershbaum et al., 1961, 1966; Murchison and Fyfe, 1966), there is an increased release of catecholamines into the blood (Kershbaum et al., 1966), a significant rise in blood glucose and a transient increase in the total platelet count both occur (Murchison and Fyfe, 1966), and platelet adhesiveness to glass is increased (Ashby et al., 1965). Platelet survival was significantly shorter and platelet turnover correspondingly greater when subjects were habitually smoking than when they were not smoking at all (Mustard and Murphy, 1963). Employing the Chandler apparatus, Engelberg (1965) found that the in vitro thrombus formation time was decreased after cigarette smoking in 34 of 60 habitual smokers.

Murphy and Mustard (1966) have reviewed this subject of smoking in relation to thrombo-embolic conditions and concluded that the relation is exceedingly complex. The many effects produced by

smoking cigarettes, such as fatty acid and catecholamine mobilisation could be factors involved in this association. Finally, it is not without interest that nicotine has been reported to induce platelet aggregation (Werle and Schievelbein, 1965).

Diabetes mellitus, glucose and platelet aggregation

Diabetes mellitus is, in some way, associated with the development of occlusive arterial disease, and the literature on this subject was cited earlier. Three reports are available which provide a possible link between this disease and platelet behaviour. Odegaard and his colleagues (1964) demonstrated a marked increase in ADP-induced platelet adhesiveness to glass in a group of twenty five patients with insulin-treated diabetes mellitus. They concluded that this was due to a plasmatic factor. Bridges et al. (1965) demonstrated an increase in platelet adhesiveness to glass when glucose was administered orally or intravenously, or when added to blood in vitro. Using an electrophoretic technique, Hampton and Mitchell (1966a) showed an increased sensitivity to ADP in three of five diabetic subjects studied.

Postoperative changes in platelet behaviour

Venous thrombosis is a serious postoperative complication and has been well documented by Warren (1953). A most authoritative, and one of the earliest publications on postoperative platelet behaviour was by Wright in 1942. Employing a technique that she devised, she showed an increase in platelet stickiness to glass with an increase in platelet numbers, beginning on the fourth day after operation and reaching a maximum on the tenth day. Extending these observations,

Bygdeman et al. (1966) demonstrated a correlation between a striking increase in postoperative platelet adhesiveness and the appearance of clinical signs of venous thrombosis. Furthermore, the maximum increase in platelet adhesiveness was detected a few days before any clinical signs of thrombosis developed. Hampton and Mitchell (1966b) studied the electrophoretic mobility of human platelets in subjects after surgical operations and found a marked increase in the sensitivity of platelets to both ADP and nor-adrenaline.

The influence of red blood cells on platelet behaviour ? please what ?

In 1960 Hellem found that little or no platelet adhesiveness could be demonstrated in platelet-rich passing through a column of glass beads, but with whole blood, a significant increase in adhesiveness was observed. The platelet adhesiveness of whole blood was, in fact, proportional to the haematocrit of the sample. It was in this very significant study that Hellem postulated the existence of a substance in red blood cells which caused platelet aggregation - his "Factor R" which was subsequently identified as ADP (Gaarder et al., 1961). Harrison and Mitchell (1966) recently considered this relationship again and concluded that in many of the in vitro tests employed to study platelet behaviour, it is possible that the abnormal platelet stickiness demonstrated in many conditions may relate to red cell abnormalities. The same conclusion was reached in another study (Hampton and Mitchell, 1966a). It remains to be proven whether this observation is an in vitro artefact or whether an interaction between red cells and platelets occurs in vivo. White cells also

effect in vitro tests of platelet aggregation (Harrison et al., 1966a).

Disseminated intravascular thrombosis

A wide variety of stimuli (Table 1) are now recognised to be able to cause platelet aggregation and these could be involved in the production of disseminated intravascular thrombosis. In recent years, the concept of disseminated intravascular coagulation has been recognised, and was the subject of a recent monograph (Mackay, 1965). Disseminated intravascular coagulation and thrombosis can probably be considered as the same phenomenon. Since the platelet contains a number of enzymes (Marcus and Zucker, 1965) which can potentially injure tissues, the question arises as to whether platelet aggregates might produce injury to the vessel wall and the surrounding tissues. Mustard and his colleagues (1964a) have claimed that ADP-induced platelet aggregates existing for a period of only three to five minutes in the coronary arteries in pigs can produce a myocardial infarct. Similarly in the kidney, transient aggregates can lead to a type of glomerulonephritis with arteriolarsclerosis and an associated hypertension (Glynn, Jorgensen and Buchanan, 1966). It has also been suggested that this mechanism, by increasing the permeability of the vessel wall to plasma constituents such as lipoproteins and proteins, might be a factor operating in the earliest stages of atherosclerosis (Mustard, Rowsell and Murphy, 1964).

In concluding this section on the platelet, the possibility has been raised that there are two platelet populations (Webber and Firkin, 1965). If this is so, the observation must have an important influence

on, and should be considered in any studies of platelet function. Furthermore, it appears that younger platelets are more adhesive than older platelets (Wright, 1942; Hirsh et al., 1966).

(iii) Blood flow and thrombosis

Although Virchow's name is the one especially associated with mechanical explanations of thrombosis, Welch (1899) states that Baillie (1793), Laennec (1819) and Davey (1839) had all previously emphasised stasis as a factor in thrombosis. Of further historical interest in Welch's discourse on this aetiological facet of thrombosis are references to Eberth and Schimmelbusch who described the effects of slowing on laminar blood flow, and to von Recklinghausen who attached more importance to turbulent blood flow in the genesis of thrombi.

Blood flow is a fundamental factor in determining the very structure of a thrombus, a point which was stressed earlier. Morphologically, thrombi formed in regions of arrested blood flow closely resemble test tube blood clots (Wessler, 1965). In Wessler's experiments, thrombosis was produced by promoting blood coagulation, and it is of considerable interest that with this mechanism, a platelet-rich thrombus, rather than a blood coagulum developed when an extra-corporeal shunt was included in the circulation (Downie et al., 1963). This seems to reinforce the opinion that sometimes, at least, the distinction between a clot and a thrombus is spurious, the difference merely being a reflection of differences in blood flow. Wessler (1955) showed that stasis coupled with a stimulus to the clotting mechanism will lead to thrombosis. In his experiments, stasis presumably

permitted the accumulation of procoagulant materials in one place, allowing a systemic tendency toward coagulation to produce local thrombosis. On the other hand, moving blood would prevent the accumulation of these materials. Whilst on this subject, the mechanical factors in venous thrombosis, which appear to be different from those in the arterial circulation, will be briefly discussed. Blood flow is slower, is uniform rather than pulsatile, and the blood pressure is lower. Venous thrombosis is restricted mainly to patients who are confined to bed and in whom venous stasis is most apt to occur. Wright and Osborn (1952) have shown by tracer techniques that posture plays an important role in the venous return from the legs; elevation of the lower extremities by as little as 10° from the horizontal doubles the venous flow rate. McLachlin and his colleagues (1960) have elaborated on this matter: using radio-opaque materials injected into the ankle veins of normal subjects they have shown that although the leg veins empty fairly quickly when the subject is horizontal, the valve pockets do not. Furthermore, rapid emptying of valve pockets can be achieved by elevating the legs 15° above the horizontal and they also demonstrated that valve pocket emptying is faster in young than in elderly subjects. At necropsy, Paterson and McLachlin (1954) and Cotton and Clark (1965) have both shown that venous thrombi develop at sites of venous stagnation. Paterson and McLachlin (1954) claimed that incipient venous thrombi in the deep leg veins form almost invariably at the apices of valve pockets. Sometimes, however, only one of a pair of valve pockets was affected, and this suggests that

something other than stasis is involved in initiating the process.

Not only the structure, but the extent of thrombosis is dependent on the type of blood flow. In the major arteries, where blood flow is fast and predominantly laminar, vessel injury produces only a thin coating of platelets, leucocytes and fibrin (Poole, Sanders and Florey, 1958), but when the flow is turbulent a typical laminated thrombus develops (Downie et al., 1963). In vitro platelet clumping is dependent on movement (Mitchell and Sharp, 1964), and Bangham (1962) has suggested that if platelets are brought together with an approach velocity of some 2 cm. per second, the normal forces of repulsion will be overcome, and the cells will then adhere. The electrokinetic response of blood platelets is modified by aggregating agents (Hampton and Mitchell, 1966c,d) and theoretically lower approach velocities would suffice when the surface charge of the platelet is modified so. Turbulent blood flow would produce much better mixing of platelets than would laminar flow.

Using extracorporeal shunts, Rowntree and Shionoya (1927), Best, Cowan and MacLean (1938) and more recently Mustard et al. (1961) and Murphy et al. (1962) have all demonstrated that the deposits which formed were not diffuse but occurred consistently at certain sites. Cinephotography was employed in these latter studies and showed that in the areas where deposits occurred the pattern of blood flow was abnormal. In the extracorporeal model, injury to the wall can be excluded and the deposits probably result from mechanical injury to the formed elements of the blood where the

flow is turbulent, when they are thrown against the wall and against each other with release, perhaps, of clumping agents such as ADP. In view of these observations, Geissenger and his associates (1962) examined the aortas of normal intact swine of various ages and found a striking similarity between the topography of thrombi occurring on endothelium, those in flow chambers, and the early lesions of atherosclerosis. Furthermore, these microthrombi were found on the endothelium of aortas from newborn animals. In the intact animal, including man, besides possible mechanical injury to the circulating cells at points of turbulence, the effects of injury to the vessel wall must also be considered. This latter may occur in two different ways. Although their interpretations differ, two groups of workers (Wesolowski et al., 1965; Texon et al., 1965) have both suggested that turbulence can directly produce arterial injury. Wesolowski and his associates (1965) suggest that one mechanism by which turbulence in the arterial tree could be involved in atherogenesis is by the production of localised areas of increased lateral wall pressure, that is, local hypertension. This effect would be enhanced in the presence of systemic hypertension; the reported association between hypertension and atherosclerosis (Kannel et al., 1961) is well known. The second possibility is that the release of substances from injured blood cells might also affect the vessel wall. Packham and her colleagues (1965) claimed to have demonstrated an increase in the permeability of the aortas of pigs to Evans blue and I^{131} albumin only at sites where there is disturbed blood flow. They concluded that this was due to an interaction between

the formed elements of the blood and the vessel wall because aortic permeability to the two test substances decreased by more than 60% when the pigs were given reserpine to deplete their platelets of serotonin.

Based on the above observations, Mitchell and Schwartz (1965) and Fox and Hugh (1966) have both proposed the following link between non-laminar blood flow, thrombosis, and atherosclerosis: at focal sites the pattern of flow is disturbed, and this assists in the collision of platelets and other cells producing a thrombus which is subsequently incorporated into the vessel wall to produce a plaque of atheroma.

Fox and Hugh (1966) suggested that the viscosity of the circulating blood is probably the main factor affecting blood flow and therefore controlling the deposition of thrombi. This aspect has been largely neglected by research workers, but Dintenfass in Australia has, for several years now, devoted his attention to rheological factors in thrombosis. His publications are many, but his views, based largely on personal observation, are summarised in a recent article (Dintenfass, 1965). His thesis is that an increase in the degree of aggregation of the red cells causes an increase in the viscosity of the blood. These red cell aggregates may plug small vessels and may also displace the platelets from the axial into the peripheral stream, where in a field of high rates of shear, platelet aggregation will occur. In turn these platelet aggregates may also plug small vessels and might initiate intravascular coagulation. Contrary to the general view, he suggests that both the cardiac infarct and the thrombus in the larger

coronary vessels are secondary to micro-thrombi in capillary vessels. His conclusions are difficult to correlate with many of the known facts, for instance, the relationship between coronary artery plaques and thrombosis, and require further investigation. An increased blood viscosity has been demonstrated in patients suffering from venous thrombosis or coronary occlusion (Dintenfass, 1964) and in diabetes mellitus (Skovborg et al., 1966). Furthermore, Dintenfass et al. (1966) showed that in menstruating women, blood viscosity was at its peak in the fourth week, rapidly decreased at menstruation, and then levelled off and remained steady till the third week. Investigators in the field of cardiovascular research are still looking for a mechanism which explains the lower incidence of arterial disease in women.

The fate of thrombi

A thrombus may resolve, embolise, or become organised. The subject of embolism is not relevant to this review and will therefore not be discussed.

Mustard and his colleagues (1964) consider that thrombosis is a "relatively frequent but insignificant clinical event". Thus, a clinically apparent thrombus represents but one end of a spectrum, and at the other extreme a "thrombus" may consist only of a microscopic platelet aggregate or a few strands of fibrin. Studies of the micro-circulation show that vessel injury produces platelet thrombi which are unstable and embolise (Honour and Russell, 1962). The mechanisms involved in the removal of thrombi have already been discussed, and it may be that interference with these mechanisms, with

consequent defective removal of mural deposits, may explain why thrombi can persist and undergo organisation. Excessive formation of thrombus material could also lead to organisation rather than resolution. The organisation of thrombi is relevant to the pathogenesis of atherosclerosis and this aspect will not be discussed.

There now seems little doubt that the organisation of mural thrombi may be one factor in the pathogenesis of atherosclerosis. That atheroma might have a thrombogenic basis was suggested originally by Rokitansky in 1852 (Fig. 1). This hypothesis received some support from Mallory (1913) and later by Clark, Graef and Chasis (1936), but gained some measure of acceptance only after the clear and convincing work of Duguid (1946, 1948) which suggested that such lesions might result from the organisation of mural thrombi. This concept has received growing support over recent years, particularly from Morgan (1956) and Mitchell and Schwartz (1965). The latter concluded from their necropsy survey that most of the histological features of human atheroma are explicable on the basis of the organisation of thrombi, stressing the contribution of platelets in addition to fibrin and red cells. Duguid (1946) and Morgan (1956) seem to have ignored the platelet despite its prominence in arterial thrombi and Duguid (1946) concluded that "collections of corpuscles such as are found in 'red' thrombi", undergo softening and account for the lipid content of atherosclerotic plaques.

Relevant to this concept of Duguid's is the observation by Magarey in 1949 that the small fibrous tags present on the mitral

Fig. 1 . Carl von Rokitansky (1804-1878). Copied from "Arterial Disease", J.R.A. Mitchell and C.J. Schwartz, Blackwell, 1965. Original from a lithograph in the Wellcome Historical Medical Museum.

and aortic valve cusps known as Lambl's excrescenses are the result of organisation of small deposits of fibrin. Their occurrence along the line of closure of the mitral valves could be due to injury at this site.

A number of attempts to confirm this aspect of arterial pathology have been made in animals, but the experimental models used in most cases are subject to a number of serious objections, including the use of blood clot rather than thrombus, and the failure to use autologous blood. One of the pioneer studies was performed by Harrison in 1948, who produced fibro-elastic lesions in the pulmonary arteries of rabbits by injecting fragmented human blood clot intravenously. Wartman, Jennings and Hudson (1951) using clots obtained from rabbit blood confirmed the fibrous, lipid-free nature of the resulting pulmonary artery lesions. Similar results were obtained by Heard (1952) using clots derived from autologous rabbit blood, by Barnard (1954) and by Thomas, O'Neal and Lee (1956). Heard (1952) found that small clots disappeared, while large clots survived long enough to become organised and converted into fibrous tissue. He speculated whether in certain circumstances fibrinolysis may be less efficient in the systemic arteries of human beings, allowing thrombi to persist long enough to become organised and converted into arterial plaques. McLetchie (1952) injected rabbits with Russell's viper venom and confirmed that fibrin underwent fibrous replacement but in addition observed that larger red mural thrombi were sometimes produced by these injections and that these became organised superficially,

but their deeper parts underwant fatty degeneration. Heptinstall (1957) utilized blood clot from hypercholesterolaemic rabbits, but found little lipid in the organising pulmonary emboli injected into rabbits on a normal diet.

In the above studies, the emboli used have been derived from either autologous, homologous, or heterologous blood clot and this differs both structurally and in composition from thrombus. By inserting a magnesium-alloy coil, or a segment of polyethylene tubing into the abdominal aorta of rabbits, Friedman and Byers (1961) succeeded in producing intra-arterial thrombi. In hypercholesterolaemic rabbits these thrombi evolved into dense fibrous intimal plaques containing lipid, often showing central degeneration and liquefaction. In a subsequent paper (Friedman and Byers, 1965) they claimed that the leakage of cholesterol rich plasma from newly formed vessels in the process of organising the thrombus contributed the lipid. Hand and Chandler (1962) studied the organisation of autologous pulmonary thrombo-emboli produced in the Chandler apparatus (Chandler, 1958). These thrombi are strikingly similar in structure to natural arterial thrombi, and these workers demonstrated that these thrombi, in the rabbit pulmonary artery, were transformed into lipid bearing fibrous intimal plaques, and that the lipid, at least in part, was derived from the phagocytosis of platelets.

Aims of the present study

In Australia, the mortality from coronary heart disease is

very high, being exceeded only by that in the United Kingdom, the United States of America and Sweden (W.H.O., 1965). Moreover, it has recently been claimed that in the period from 1950 to 1962 in Australia, mortality from this cause has increased considerably in men (Reader and Wynn, 1966). Thrombosis and vascular disease constitute one of the major causes of morbidity and mortality in the world today. This problem is still far from clearly understood and this is reflected by the length of the review which has been presented as an introduction relevant to the work upon which this thesis is based.

One possible interpretation of the data available on this subject of thrombosis and arterial disease, is that a primary alteration in the circulating blood occurs, presumably governed by environmental factors, and this results in the formation of thrombi at certain sites in the arterial system, these sites being determined by the pattern of blood flow. These thrombi then organise into wall plaques in the manner suggested by Duguid (1946). Whether the generalised change in the circulating blood is due to hypercoagulability or to an increased "stickiness" of platelets has not been clearly defined. Moreover, the evidence in favour of a generalised change is by no means conclusive. The thrombogenic hypothesis is still discounted by many workers on the basis that it has not been proven experimentally, and secondly, that it cannot possibly account for the development of the fatty gruel within the centre of the plaques.

The studies contained in this thesis have been directed at the solution of the problems mentioned in the last paragraph.

(i) In part II the findings from a study of the organisation of thrombi of varying composition in the pulmonary arteries of rabbits are described, and compared with the changes found during the organisation of thrombi in an extravascular site, the anterior eye chamber of the rabbit. Because of the alleged relationship between abnormal lipid metabolism and atherogenesis the influence of dietary induced hypercholesterolaemia and lipaemia on the lesions resulting from the organisation of thrombi was also investigated. Finally, the ultra-structural changes occurring during the organisation of thrombi were examined.

(ii) Recent information pertaining to the effect of adenosine nucleotides on platelets has re-focussed attention on the importance of platelet aggregation in the pathogenesis of thrombosis. Employing a spectrophotometric technique the effects of nucleotides, catecholamines, fatty acids and methyl xanthines on platelet aggregation have been investigated. This aspect is contained in part III of this thesis.

(iii) The studies included in part IV were designed to determine if the blood of patients with, after, or prone to arterial or venous thrombosis differs from the blood of healthy control subjects, using an in vitro system never before employed for this purpose. Particular attention was directed to platelet behaviour in these conditions.

PART II

THE FATE OF THROMBI

TABLE 3

DISTRIBUTION OF ANIMALS RECEIVING EITHER PLASMA OR WHOLE BLOOD PULMONARY THROMBO-EMBOLI GROUPED ACCORDING TO THE TIME AFTER OPERATION AT WHICH THEY WERE KILLED.

Time after operation		Type of thrombus# introduced	
		Plasma	Whole blood
Days	1	C5*	
	2	13 49	
	3	42 48	
	4	41 46 47	
Weeks	1	25 31 39 40 43 44 45	72 73 74
	2	34 35 36 37 38	69 70 71
	3		66 67 68
	4	28 29 30 32 33	63 64 65
	6	23 24 26 27	60 61 62
	7	21 22	
	8	18 19	
	11	15 16	
	12	12	57 58 59
	14	2 3 4 9	
	15	8 1 5 6 7	
	18	10 11 14 17 20	

* Figures indicate experiment number.

#NOTE: According to accepted terminology a "thrombus" is a solid mass of blood which forms in vivo, whereas the word "clot" is applied to the solid material which forms in vitro. To emphasise the resemblance of the clot produced in the Chandler apparatus to a natural thrombus, the term "artificial thrombus" seems to be preferable - for the sake of brevity, however, the author has used the term thrombus.

PART II - THE FATE OF THROMBI

Because the "thrombi" produced in the Chandler apparatus are so similar to those which occur naturally, this was considered as an ideal model with which the detailed organisation and fate of thrombi of differing composition, in different sites, and under different conditions could be studied over a wide range in time. The organisation of autologous whole-blood and plasma pulmonary thrombo-emboli prepared in this apparatus, was studied in normocholesterolaemic rabbits, and compared with the changes found during the organisation of plasma thrombi inserted into the anterior eye chambers of rabbits. In a second experiment, the influence of hypercholesterolaemia upon organisation is reported, and finally, the ultrastructural features of the organisation of plasma pulmonary thrombo-emboli are described.

Experiment 1: The organisation and fate of autologous pulmonary thrombo-emboli in normocholesterolaemic rabbits, and a comparison with the fate of plasma thrombi in the anterior eye chamber

1. Materials and Methods

Seventy four adult male rabbits of mixed breed, weighing from 2.0 to 3.5 Kg. and fed ad lib. on a standard rabbit pellet diet (40.8% bran, 41.5% pollard) were used. Of these, 6 were retained as controls, and the remaining 68 had pulmonary thrombo-emboli introduced. In 50 of the rabbits, the thrombo-emboli were derived from autologous plasma, and in 18 from whole blood (Table 3). Plasma thrombi were introduced into the anterior eye chambers of 39 rabbits, 38 of which were in the group of 50 rabbits embolised

TABLE 4

DISTRIBUTION OF ANIMALS WITH PLASMA THROMBI INSERTED INTO THE EYE CHAMBER, GROUPED ACCORDING TO THE TIME AFTER OPERATION AT WHICH THEY WERE KILLED.

Time after operation		Experiment number
	2	13 49
Days	3	48
	4	46 47
	1	31 39 40
	2	35 37 38
	4	32 33
	6	23 24 26 27
	7	21 22
Weeks	8	18 19
	11	15 16 10A
	12	12
	14	2 3 4 9
	15	1 5 6 7 8
	18	10 11 14 17 20

with plasma thrombi (Table 4).

Operative procedure

Each rabbit was anaesthetized with an initial dose of 1.0-1.5 ml. of pentobarbitone sodium introduced slowly through a Gordh needle inserted into the right marginal ear vein. Further 0.2 ml. amounts were given as indicated. The anaesthetized rabbit was placed on its back on an operating table and secured. Local anaesthetic, 1 ml. of 2% procaine, was given subcutaneously and the right external jugular vein exposed. The vein was opened between ligatures and a polyethylene cannula with an internal diameter of 1.6 mm. and containing an occluding stilette made from plastic covered wire was inserted towards the heart and secured in situ. Blood was withdrawn into a siliconized glass syringe, and the siliconized stilette reinserted. Nine millilitres of blood was added to 1.0 ml. of 3.8% trisodium citrate in a siliconized glass centrifuge tube and mixed by gentle inversion. Artificial thrombi were prepared from either citrated whole blood or plasma. Plasma was prepared by centrifuging at 350 g. for 15 minutes. Aliquots (1.5 ml.) of citrated whole blood or plasma were introduced into loops of polyvinyl chloride tubing and recalcified by the addition of 0.15 ml. of M/4 calcium chloride. Each tube was closed, placed on the rotor of the modified Chandler apparatus and rotated at a linear velocity of 340 cm./min. until 10 minutes after a change in the angle of the advancing column of blood or plasma had occurred. The Chandler apparatus is discussed in detail in part IV and is illustrated in Fig. 67 In each experiment three plasma or whole blood thrombi were prepared

simultaneously. These were emptied into a sterile Petri dish containing sterile 0.9% saline. The tail, consisting of clot or a loose fibrin meshwork was removed, and the remaining head of each thrombus was divided into segments 5-8 mm. long. These segments of thrombus totalling 4-5 cm. in length were suspended in 5 ml. of sterile 0.9% saline and slowly injected via the external jugular cannula (Fig. 2). The cannula was removed and the vein ligated. After dusting with penicillin powder, the wound was closed with 12 mm. Michel clips, and the area sealed with a skin of Parlodion (cellulose nitrate in equal volumes of ethyl alcohol and ether). Sterile precautions were enforced throughout the procedure.

Insertion of thrombi into the anterior eye chamber

After several drops of 1% amethocaine had been applied to the left eye, the anterior chamber was opened with a tenotomy knife, and one or two 5 mm. segments of plasma thrombus were inserted with a pair of fine ophthalmic forceps. These were passed with a fine probe around the margin of the anterior chamber to a point opposite the incision and wedged into the irido-corneal angle, according to a technique originally described by Higginbotham (1957). The tenotomy incision was not sutured, and penicillin ointment was placed in the conjunctival sac. A plasma thrombus in situ in the anterior chamber is shown in Figure 3.

Preparation and examination of the lungs and eyes

The time after operation at which the rabbits were killed and the number of animals in each group is shown in Tables 3 and 4.

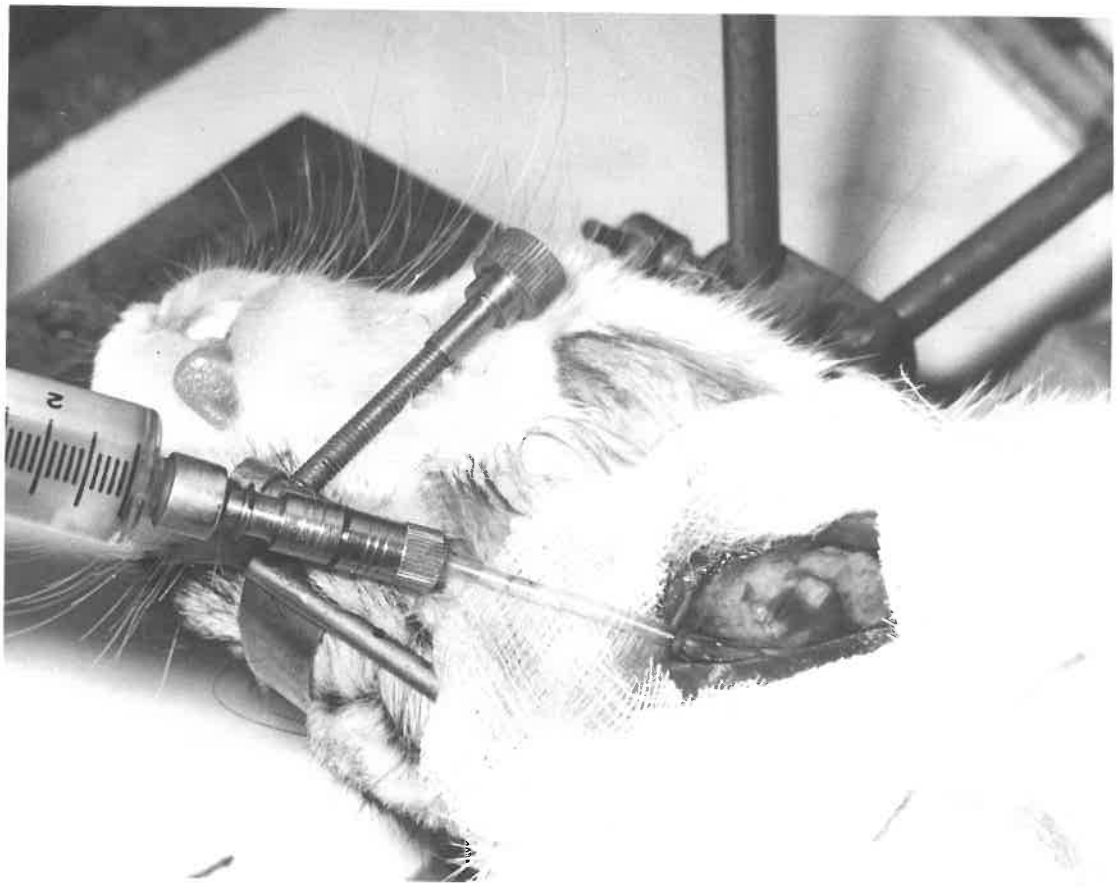
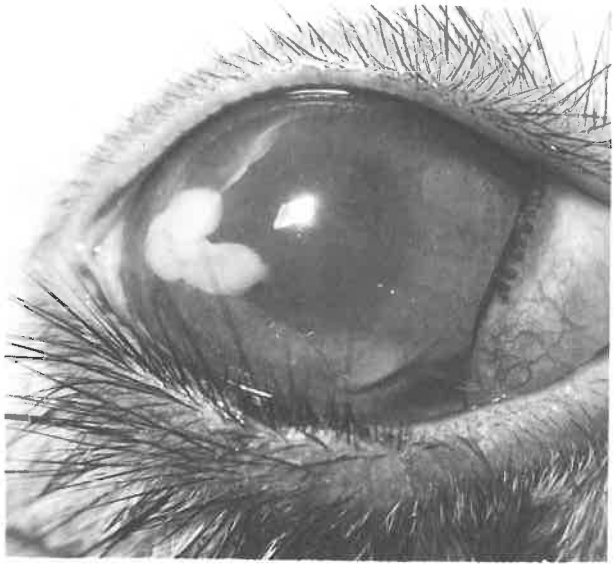
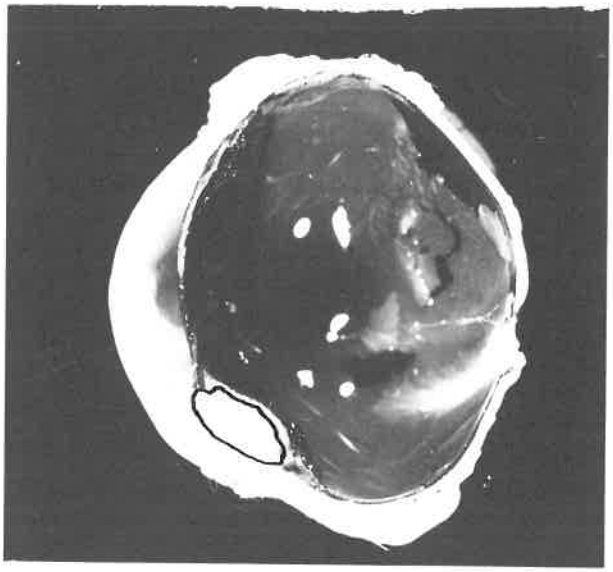


Fig. 2. Plasma thrombi suspended in saline in the syringe are being injected into the rabbit via the cannula in the external jugular vein.



A. Photograph taken at the completion of the operation.



B. A bisected eye with a plasma thrombus (outlined) in the anterior eye chamber. Three days after operation.

Fig. 3. A plasma thrombus in the anterior eye chamber of a rabbit.

The heart and lungs were removed en masse, and the lungs inflated through the trachea with Baker's buffered calcium-formol-saline (Culling, 1957). All specimens, including the eyes were fixed in Baker's calcium formol. After fixation the lungs were separated from the heart and sectioned at 1-2 mm. intervals from the periphery to the hilum. Between 15-18 blocks numbered in sequence from the hilum to the periphery were obtained from each lung. Both surfaces of each block of tissue were examined under magnification for macroscopic lesions, and a minimum of 5 blocks from each lung was selected for histological examination. Routinely six serial 5 μ paraffin sections were prepared from each block, three of which were stained with haematoxylin and eosin (H. + E.) and three with Gomori's elastic stain. In many cases the blocks were subsequently sectioned serially, and in addition a number of thin 2-3 μ sections were prepared. Additional stains employed included periodic acid-Schiff (P.A.S.), phosphotungstic acid-haematoxylin (P.T.A.H.), toluidine blue, alcian blue, Masson's trichrome, picro-Mallory (Carstairs, 1965), Prussian blue, and von Kossa. Selected blocks were embedded in polyethylene glycol (Carbowax 1500) and stained for lipid with Scharlach R. A total of 3,094 blocks of lung tissue were processed and some 42,000 sections were examined (Appendix A).

2. Results

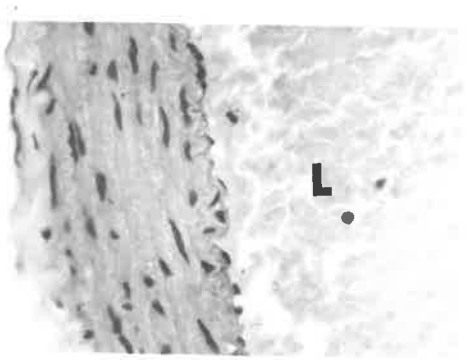
Histology of the normal pulmonary artery

In order to assess the significance of experimentally-induced lesions it is first necessary to define the morphology of the pulmonary

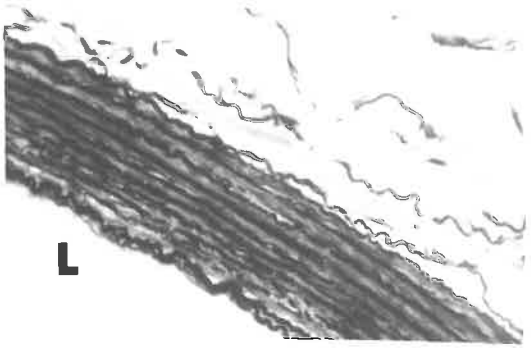
arteries in normal healthy rabbits. As the experimentally-induced lesions were located in the large to medium sized pulmonary arteries, this description will be confined to such vessels with an external diameter ranging from 0.4 to 2.0 mm.

The first branches of the main pulmonary artery were predominantly of the elastic variety, with between 7-10 circularly arranged medial elastic laminae alternating with circularly orientated smooth muscle cells and a little collagen (Fig. 4A, B). Subsequent branches contained a diminishing quantity of medial elastic tissue, in finer laminae, and relatively more smooth muscle. Medium sized branches of the order of 0.4 mm. external diameter were found to possess between 6-10 layers of medial smooth muscle cells, and a small but variable quantity of medial acid mucopolysaccharide was seen. The adventitia was between 50-75 percent as thick as the media and contained coarse irregular collagen fibres and obliquely orientated coarse elastic fibres (Fig. 4B). Apart from scattered fibroblasts and an occasional lymphocyte no other cells were seen. The vasa vasorum were inconspicuous. A well defined coarse and fairly continuous external elastic membrane was prominent.

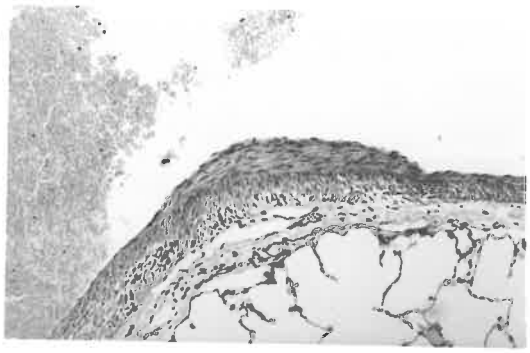
The normal intima consisted of a single layer of endothelial cells immediately beneath which was seen a thick wavy continuous internal elastic lamina in which no fenestrations were apparent (Fig. 4A). Occasionally a few spindle cells and a minimal amount of collagen were interspersed between the endothelium and the internal elastic lamina. Focal splitting of the internal elastic



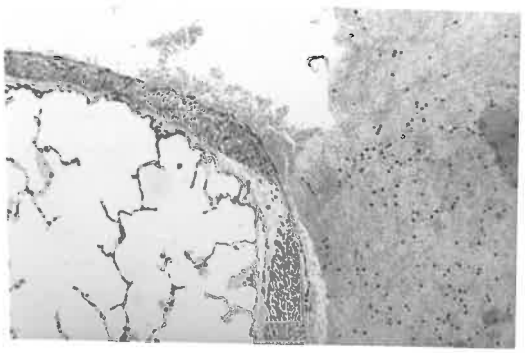
A. H. & E. x 375



B. Gomori elastic. x 375



C. H. & E. x 85



D. H. & E. x 85

Fig. 4. Morphological aspects of the normal pulmonary arteries of the rabbit. L = lumen of the vessel.

membrane was frequent, but occasionally a circumferential reduplication was present.

Medial defects were prominent at branching points and bifurcations. These consisted essentially of a partial or complete loss of medial muscular and elastic tissue (Fig. 4D). An almost universal finding was the intimal cushion or pad, also located at bifurcations and branching points. These consisted predominantly of smooth muscle cells most of which were orientated with their long axis in the direction of blood flow (Fig. 4C); in addition there was a small amount of collagen and some fine elastic-staining fibres. The internal elastic lamina beneath these cushions invariably showed splitting.

Apart from the intimal cushions and medial defects, no other lesions involving either the intima or media were apparent in a large number of sections, and in particular no thrombi were observed.

Morphology of the artificial thrombi produced in the Chandler apparatus

Both whole blood and plasma thrombi consisted of a head, in which platelet aggregates were prominent, and a tail, devoid of platelets, which was essentially nothing more than a blood clot. On serial section the whole blood thrombi contained a significantly greater number of platelets than did the plasma thrombi. Despite the prominence of the platelets at the head of these thrombi fibrin was still a major component. Whole blood thrombi also contained copious red cells and leucocytes; the latter were characteristically arranged around the margins of the platelet clumps.

The fate of plasma thrombi

The earliest observations were made 1½ hours after embolization in one rabbit (C5). A virtually occluding thrombus (Fig. 5) had been arrested at a point of bifurcation. The vessel wall was dilated and appeared thinned. For part of its circumference the thrombus was in close contact with the endothelium, but elsewhere a narrow gap existed, and here the free surface of the embolus was covered by a secondary thrombus consisting predominantly of platelet aggregates (Fig. 6); this was observed only during the first week after embolization. Platelets were also seen within some of the crevices in the embolus, while in others perhaps where flow was stagnant only red cells were observed.

Two other findings of note were also present at this early stage. In H.+E. sections platelets within the embolus were noticeably paler than the platelets on the surface, suggesting that the former were already partially degenerate. The second feature was the peripheral invasion of the thrombus by eosinophilic polymorphonuclear leucocytes (Fig. 7). This was the first demonstrable cellular response to the presence of the thrombo-embolus.

Forty eight hours. Though variable, by 48 hours the peripheral polymorphonuclear leucocytic invasion had become prominent (Fig. 8A). At this stage the pseudo-eosinophil, a cell comparable to the polymorphonuclear leucocyte of man, was the predominant cell. The thrombus had assumed a spongy appearance at its

Fig. 5. A plasma thrombus $1\frac{1}{2}$ hours after embolisation.

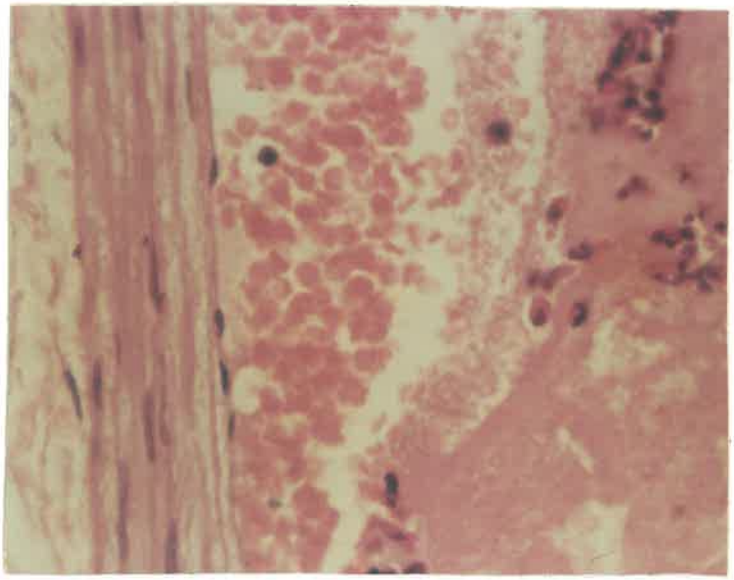
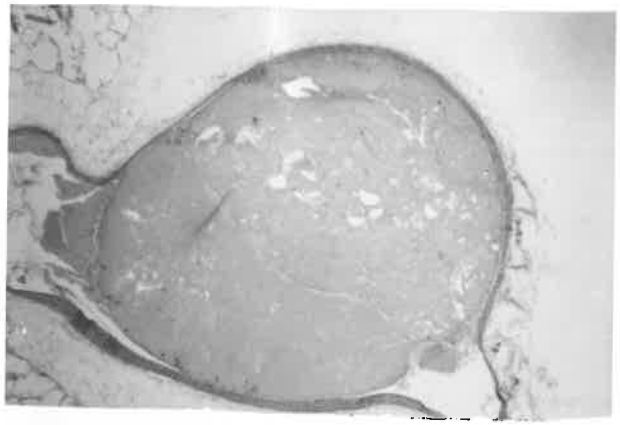
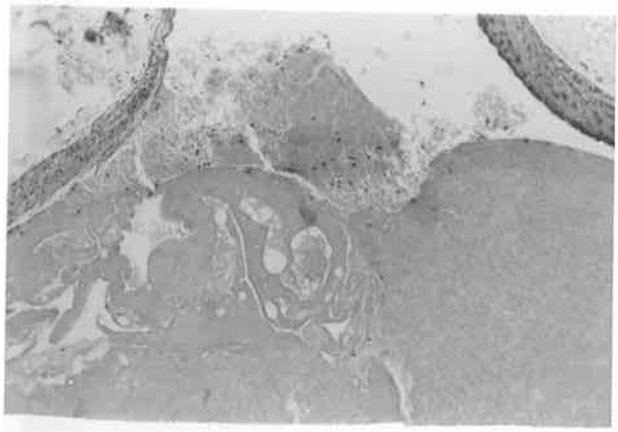


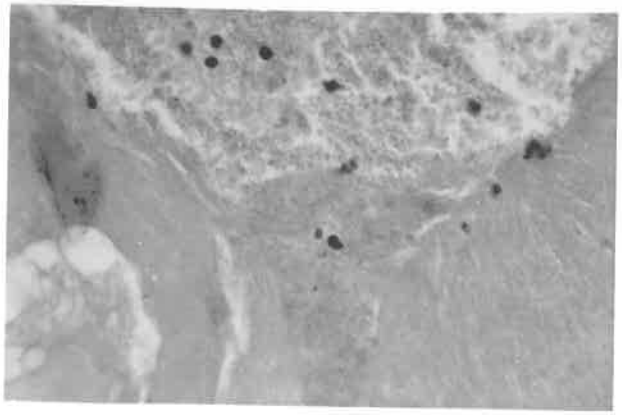
Fig. 7. Eosinophilic polymorphonuclear leucocytes in the periphery of the thrombus $1\frac{1}{2}$ hours. H. & E. x 510.



A. x 25.

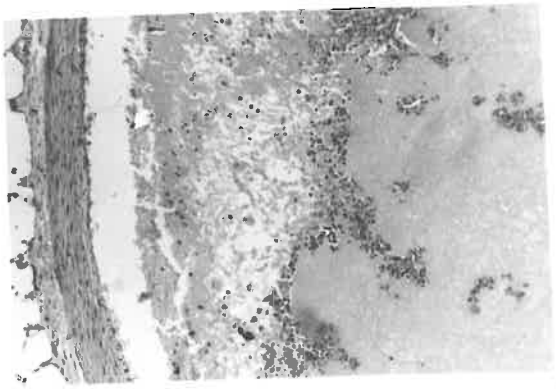


B. x 95.

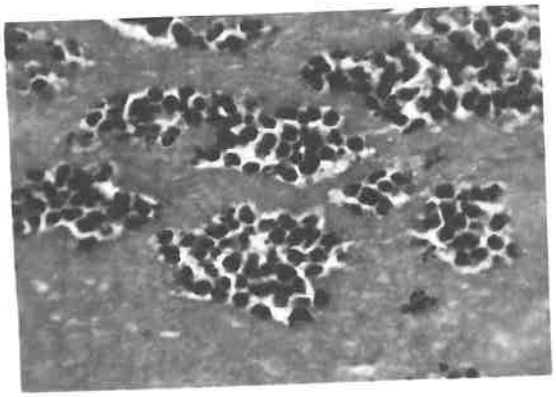


C. x 430.

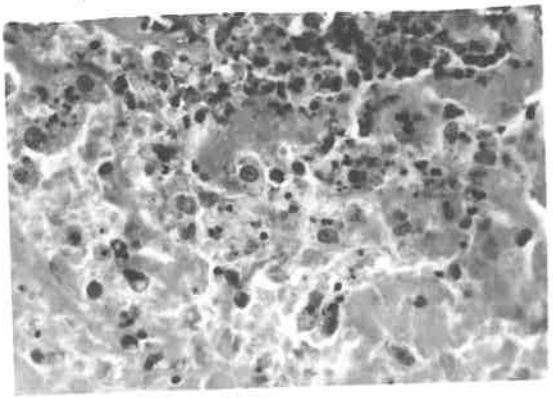
Fig. 6. A secondary thrombus consisting predominantly of platelets, on the surface of the thrombo-embolus. H. & E.



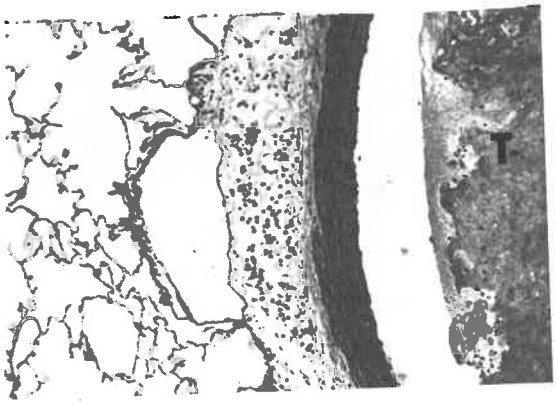
A. H. & E. x 85.



B. H. & E. x 375.



C. H. & E. x 375



D. T = thrombus
Gomori elastic. x 85.

Fig. 8. Illustrating various aspects of the cellular response to the presence of a thrombo-embolus in the arterial lumen.

periphery, with clear spaces near and around the polymorphonuclear leucocytes indicating that these have thrombolytic or fibrinolytic properties (Fig. 8A,B). Within the thrombo-embolus, degeneration and degranulation of the polymorphonuclear leucocytes had already commenced (Fig. 8C). Leucocytes were also present in the arterial wall beneath the endothelium and especially in the adventitia where some lymphocytes were also noted (Fig. 8D). When the thrombus was occlusive, the adventitial vasa vasorum were congested and dilated, but if non-occlusive, congestion and dilatation were minimal or absent.

A striking change in the arterial wall beneath and also for a short distance proximal and distal to the embolus was a marked increase in the amount of intercellular material (Fig. 9). This appeared basophilic in H. +E. sections and was alcian blue-positive and metachromatic with toluidine blue. In the media this material caused wide separation of the muscle fibres (Fig. 9), and both muscle and elastic tissue degeneration was evident. The presence and extent of this intercellular material showed no constant relationship to the degree of luminal occlusion.

Shortly after the leucocytic response, a second population of cells was seen to enter the thrombo-embolus (Fig. 10A). This mononuclear population appeared mixed, including both lymphocytes and monocytes, and a third cell type, with a nucleus larger than that of the lymphocyte, and having a loose chromatin network and one or two prominent nucleoli (Fig. 10C). These large cells on appearance

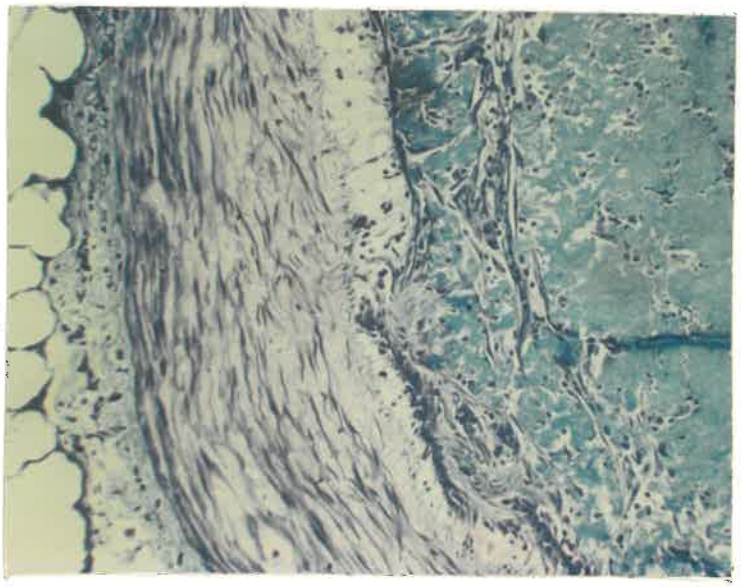
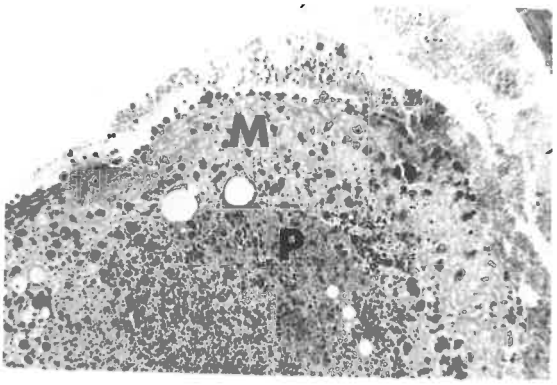
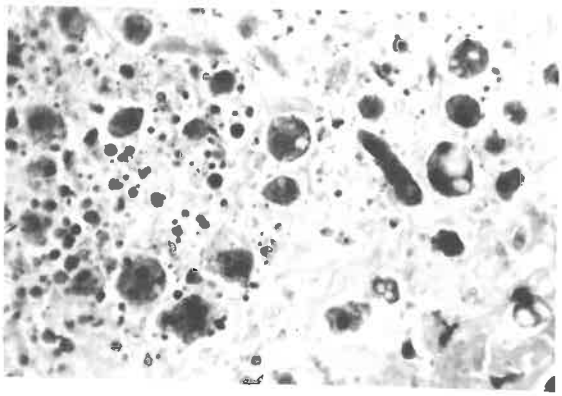


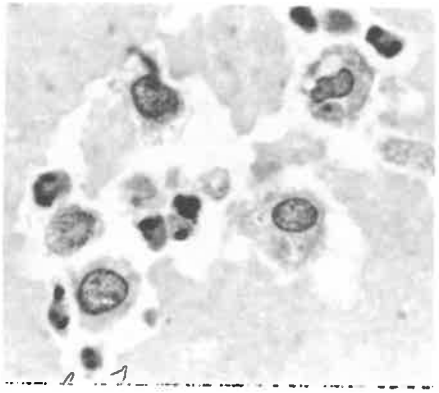
Fig. 9, Showing a marked increase in the amount of metachromatic intercellular material in the media of the vessel wall. Also nøte the metachromatic material in the thrombus associated with fibroblastic activity. Toluidine blue. x 110.



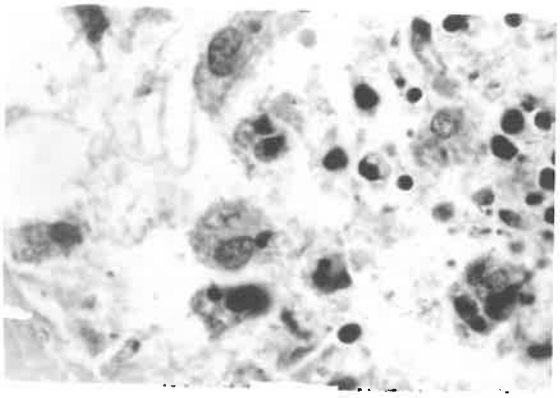
A. P = polymorphs and M = mononuclear cells. x 90.



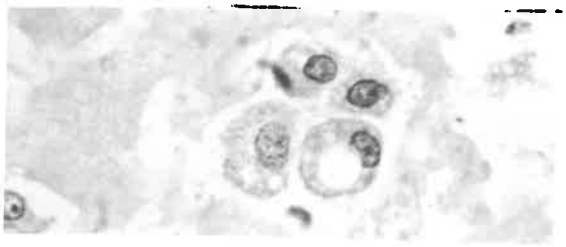
B. x 395.



C. x 830.



D. x 830.



E. x 830.

Fig. 10. Mononuclear cells within the thrombus, and phagocytosis of polymorphonuclear cell debris (B. and D.) and platelets (E.). H. & E.

could be derived from either lymphocytes or monocytes. They increased in number, and assumed phagocytic properties, becoming transformed into macrophages. In spite of this number increase, mitotic figures were infrequent in these large mononuclear cells.

Phagocytosis was first evident at 48 hours, when nuclear and other debris from degenerate polymorphonuclear luecocytes was observed within the macrophages (Fig. 10B,D). Plate phagocytosis was observed at this time, but it was often difficult to demonstrate convincingly because of platelet swelling and vacuolation after ingestion, thus making their identification difficult (Fig. 10E).

Four days. In H.+E. sections numerous scattered foamy macrophages were present by the fourth day. Carbowax-sections stained with Scharlach R revealed lipid droplets in these cells (Fig. 11). At this stage a giant cell reaction was occasionally noted within the embolus (Fig. 12). The similar appearance of the nuclei of the macrophages and the giant cells suggested a common origin.

At 3 days after embolisation a layer of flattened cells was seen, beginning to cover the unattached or free surface of the embolus. This cell layer appeared continuous with the endothelium of the vessel wall at the margins of the thrombus (Fig. 13) where endothelial proliferation was evident. By 4 days this process of endothelialization was well advanced and in the plane of many sections was complete.

Another mechanism might also contribute to the endothelialization

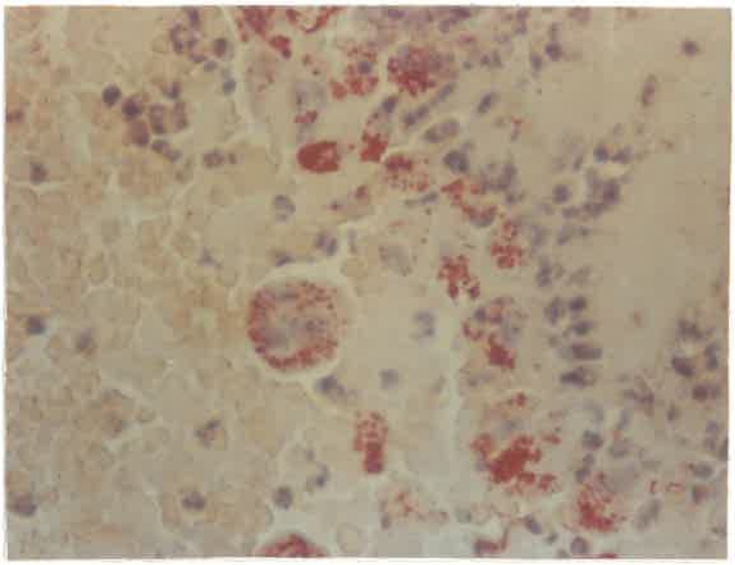
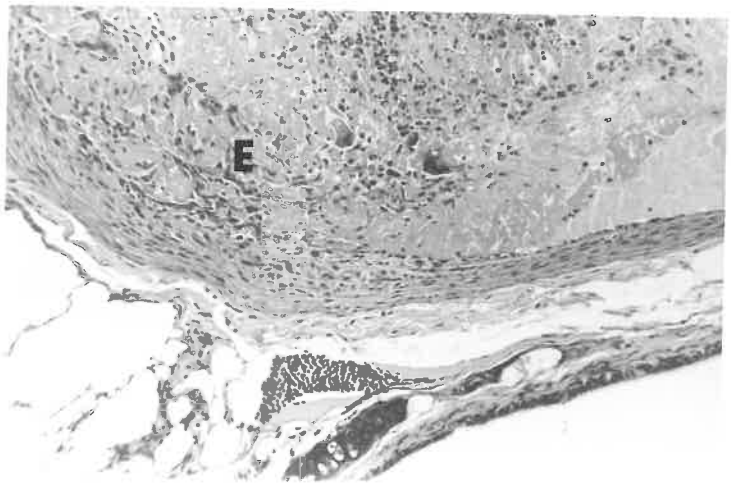
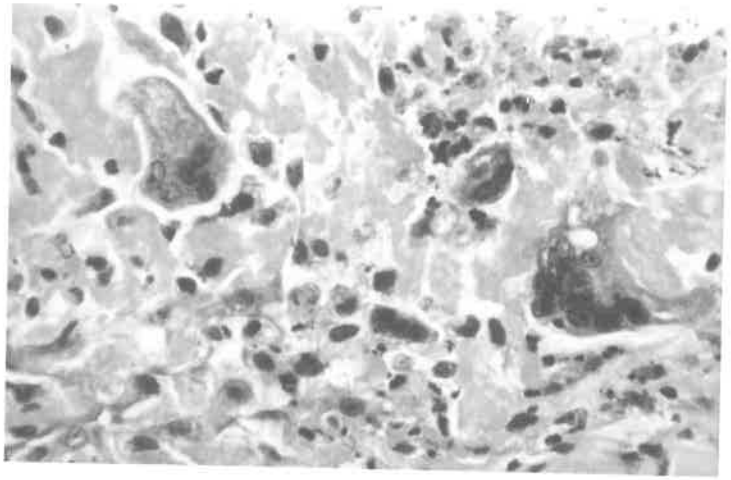


Fig. 11. At 4 days after embolisation. Numerous macrophages containing lipid droplets. Scharlach R. x 510.

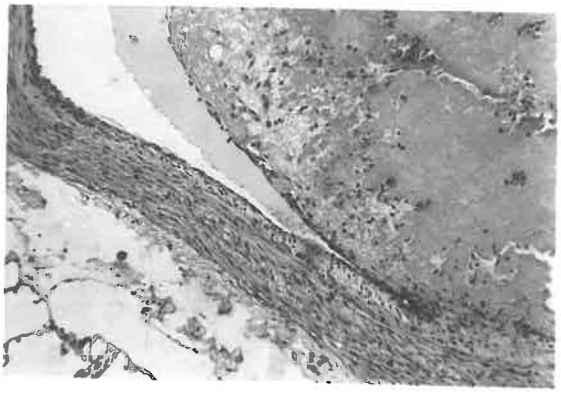


A. x 110.

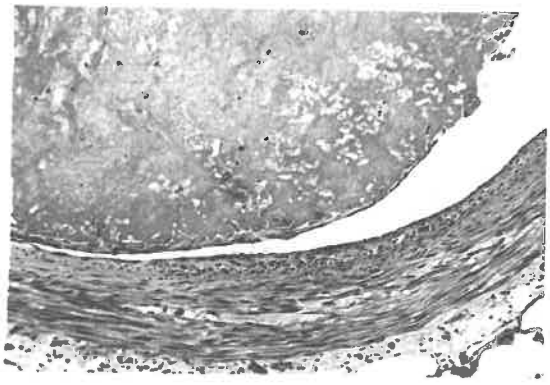


B. Higher magnification of part of Figure 12A. x 510.

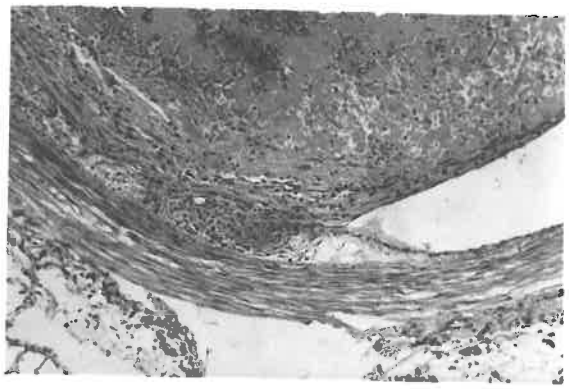
Fig. 12. Giant cell reaction within the embolus. Figure 12A. also shows proliferating endothelial cells (E.) invading the base of the thrombus. H. & E.



A.



B.



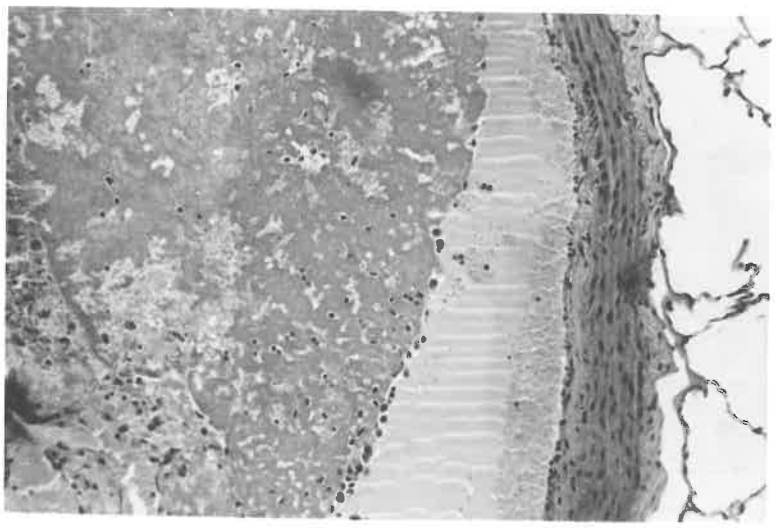
C.

Fig. 13. Endothelialisation of the surface of the thrombus by the vascular endothelium. H. & E. x 90.

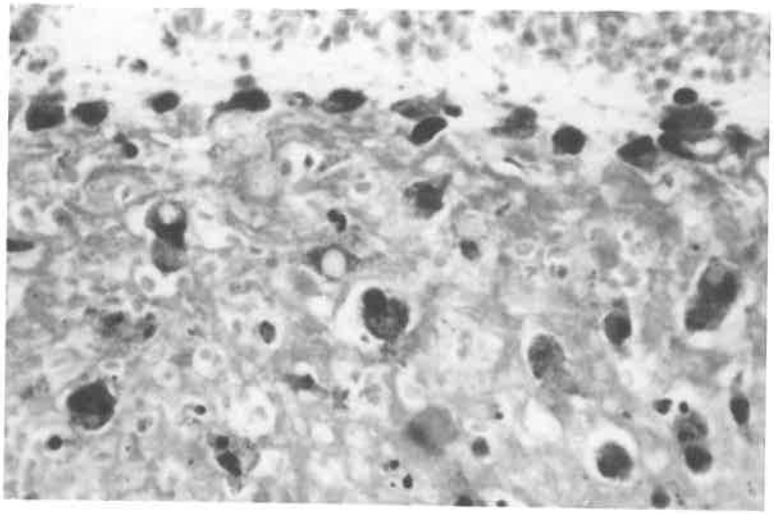
of the thrombus surface. Cells, some becoming flattened, were seen on the surface at points far removed from the margins where endothelial proliferation was noted (Fig. 14). A mononuclear origin for these cells was suggested by the presence of forms intermediate between circulating mononuclears and flattened spindle cells. After the third day spindle cells were also appearing within the thrombus. Like the "endothelial" cell covering the thrombus surface, these cells also appeared to have two possible origins. Again forms intermediate between rounded mononuclear cells and the spindle cells were observed suggesting that the circulating mononuclears might transform into spindle cells (Fig. 15A). This mechanism, however, appeared to play only a minor role in the genesis of spindle cells and fibroblasts, and a more important source was the proliferating "endothelial" layer on the thrombus surface (Fig. 15B,C) and at the base of the thrombus (Figs. 15D and 12A).

Accompanying this "endothelial" cell invasion of the thrombo-embolus, a basophilic alcian blue positive change in the immediate environment, associated with the formation of fine collagen and elastic staining fibrils was observed (Fig. 9). Surrounding some of these invading spindle cells a clear zone was noted, suggesting that these cells have a fibrinolytic property (Fig. 16A). No definite phagocytosis by these cells was discerned.

New vascular channels began to form at 3-4 days. Some of the invading "endothelial" cells were seen to line spaces and crevices (Fig. 16B). Other channels resulted from solid

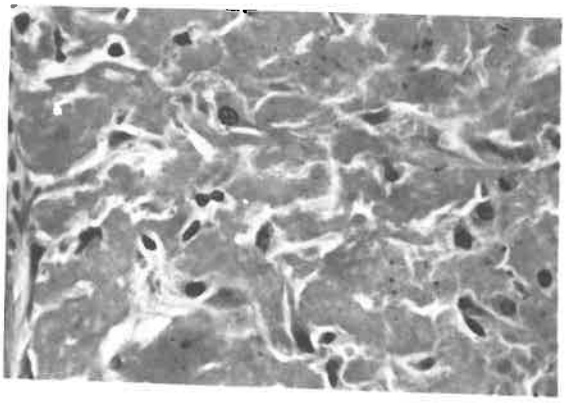


A. x 120.

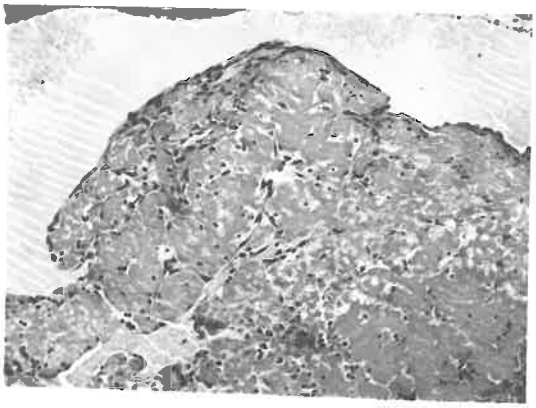


B. x 540.

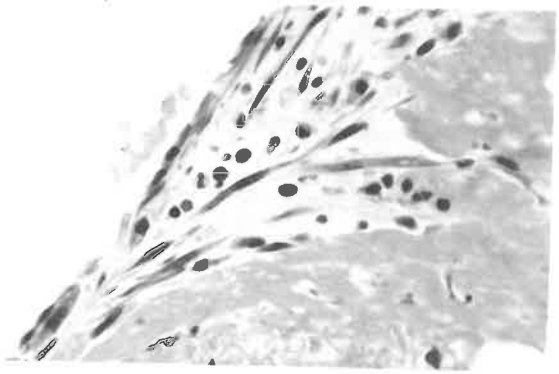
Fig. 14. Flattened mononuclear cells on the surface of the thrombus. H. & E.



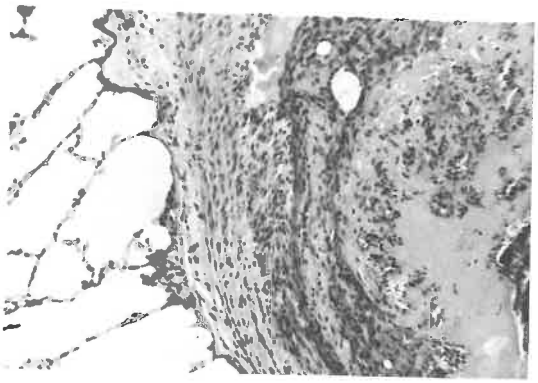
A. x 395.



B. x 90

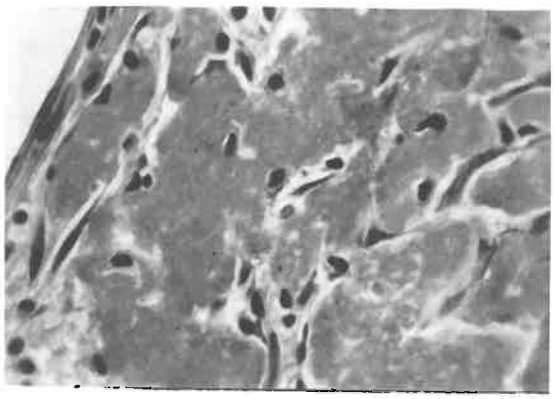


C. x 395.

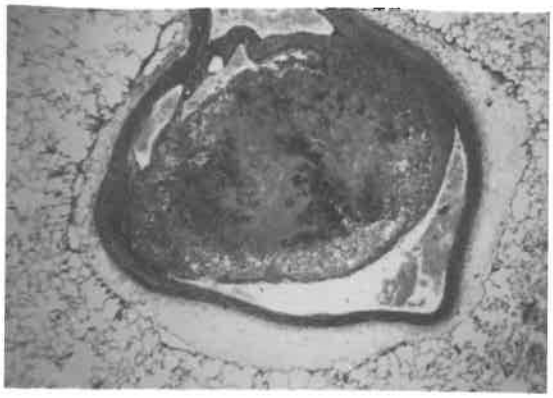


D. x 90.

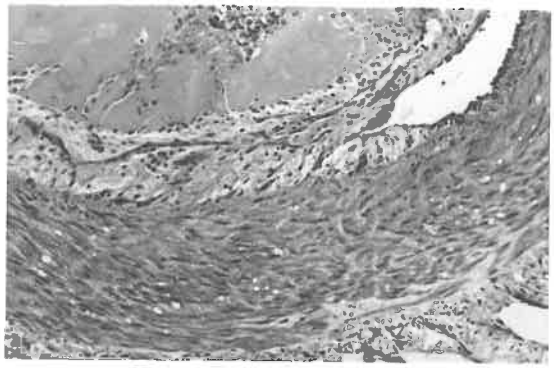
Fig. 15. Illustrating the two origins of spindle cells within the thrombus. H. & E.



A. x 395.



B. x 20.

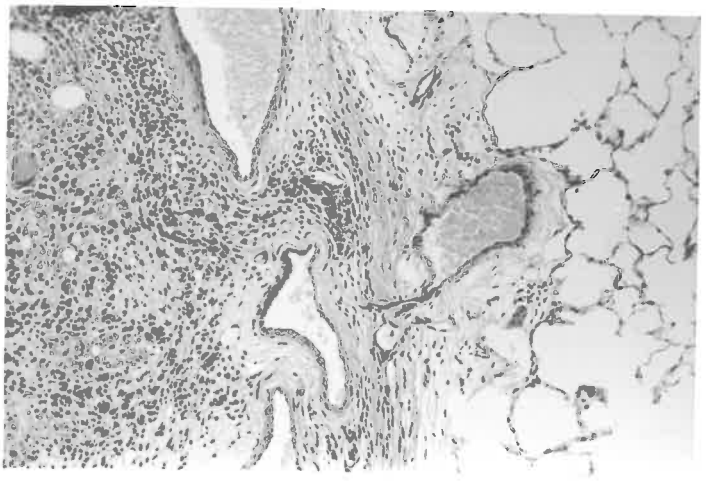


C. x 90.

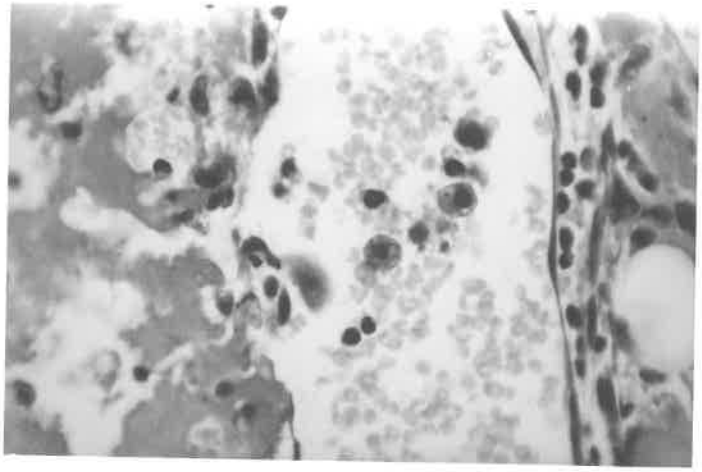
Fig. 16. A. illustrates the fibrinolytic activity of endothelial cells. B. and C. illustrate the two methods by which vascularisation of the thrombus occurs. H. & E.

endothelial buds which subsequently canalized (Fig. 16C). Characteristically these new channels were seen to communicate with the lumen at the thrombus margins (Fig. 16C). Definite communication with adventitial vessels was rarely observed, and was usually found to occur at a branching point. The media beneath eccentrically attached thrombi was often atrophic and occasionally vessels were seen penetrating this atrophic media (Fig. 17A). Foam cells were often seen in the lumina of the larger new vascular channels (Fig. 17B) and some appeared to be in transit through the endothelial lining, which itself showed no foam cell changes with the light microscope.

This picture of the early organization is the composite of a number of experiments and a very large number of sections. In some experiments, however, there was a marked variation from this general theme. Whenever the thrombus remained completely occlusive, then the process of organization was retarded. Retardation was also observed in one rabbit which had massive infarction of the lower lobes of both lungs. The most dramatic variable, however, was the intensity of the polymorphonuclear response. The two extremes of this response are depicted in Figures 18 and 19. This difference appeared to be related to variations in the structure of the thrombo-emboli. Copious platelet aggregates were always present in those thrombi in which the response was greatest, while in those where it was minimal or absent, the thrombus consisted predominantly of fibrin with few or



A. x 110.



B. x 490.

Fig. 17. New vascular channels within the organising thrombus. H. & E.

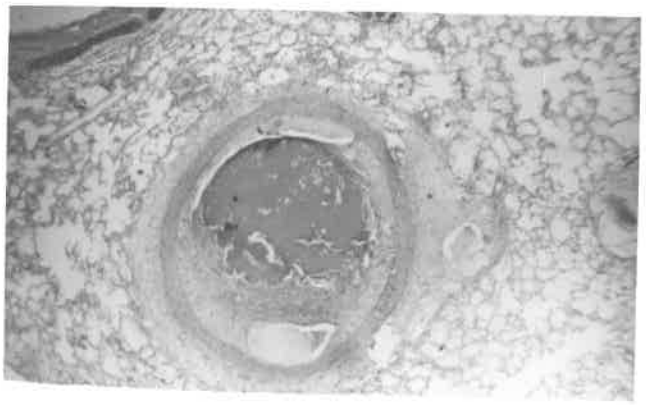


Fig. 18A. x 25.

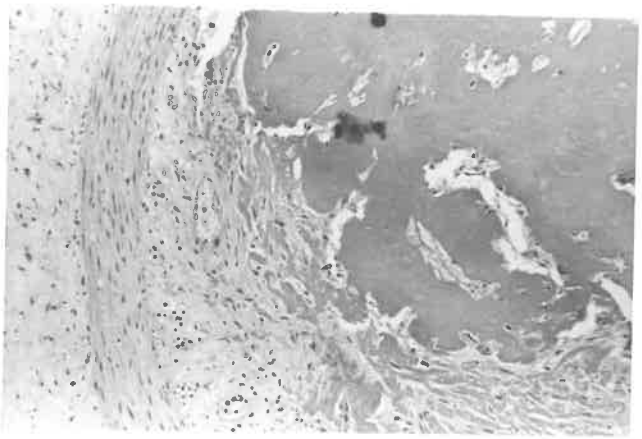


Fig. 18B. x 100.

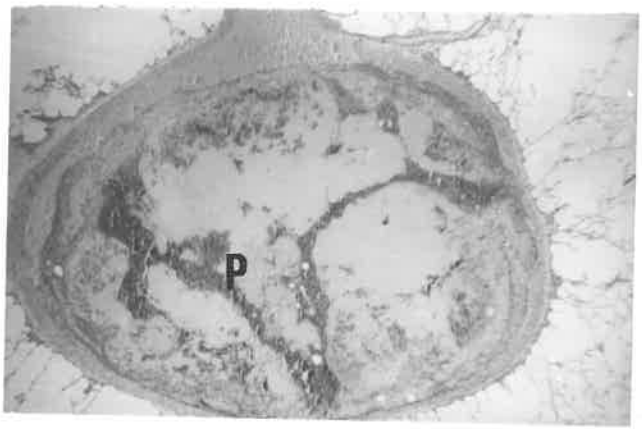


Fig. 19. P = polymorphs. x 25.

Figures 18 and 19 illustrate the two extremes of the variable polymorphonuclear response. H. & E.

no identifiable platelet masses. This observation raises the possibility that a leucotactic factor might be released from degenerating platelets within an organizing thrombus.

One week. Some of the thrombi were still almost occlusive, while others had retracted. Endothelialization was complete in most sections and the vessel wall showed a variable excess of acid mucopolysaccharide. Most thrombi were attached to only a part of the wall of the vessel, thereby producing eccentric lesions, but in a few instances the attachment was more extensive producing a concentric lesion. The media beneath eccentric lesions was often atrophic.

Two weeks. Figure 20 illustrates the progressive organization which has occurred by 2 weeks. At this stage lesions were found in 24 blocks from the 5 animals in this group. Only one of these lesions was still occlusive, the thrombus being extensively calcified. This was also the only instance in which metachromasia of the vessel wall was still persisting.

Of the 24 blocks with lesions at 2 weeks, only 3 possessed lesions with a significant foam cell reaction, the remaining blocks showing fibrous intimal lesions or residual thrombus undergoing peripheral fibrosis. The fibrous intimal plaques contained a variable amount of elastic tissue. On only one occasion was haemorrhage into a plaque seen. Not infrequently the lesions were polypoid, projecting into the arterial lumen.

Four weeks. At 4 weeks lesions were present in 20 blocks. A

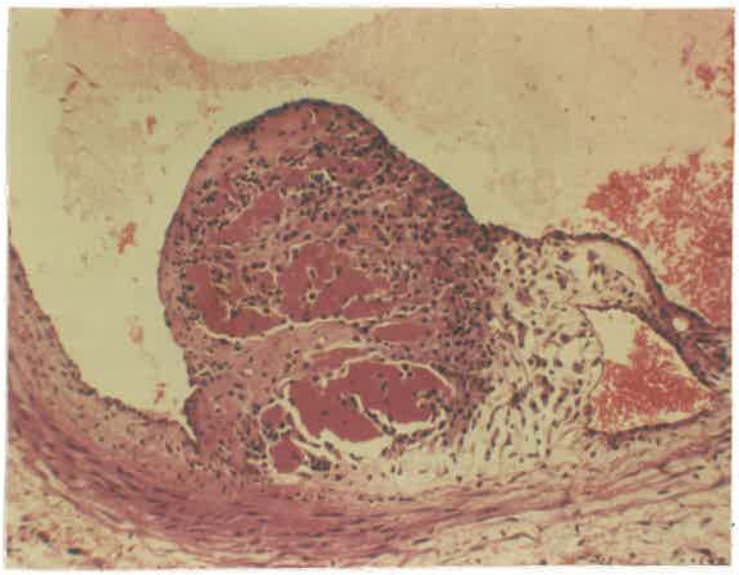


Fig. 20. At two weeks after embolisation. Residual thrombus can still be seen. H. & E. x 110.

representative lesion, shown in Figure 21 was predominantly fibrous but occasional foam cells (Fig. 22A) and lipid cysts were present. One polypoid lesion, however, consisted almost entirely of foam cells (Fig. 22B, C) but serial sections through this plaque revealed that the edges were exclusively fibrous. Two blocks, all from the one animal (33) showed lesions containing considerable calcification. A feature of note in the plaque shown in Figure 21 was the presence of a well-developed layer of smooth muscle cells in the walls of the new vascular channels.

Several of the lesions were still polypoid at 4 weeks, and occasionally an interlacing bridge of fibrous tissue completely traversing the lumen of the vessel was seen (Fig. 22D).

Six to eight weeks. Of the 8 animals in this group, lesions were found in only 16 blocks. The plaques were predominantly small and fibrous (Fig. 23A, B) most being discernible only with a microscope. Two, however, were noticeably larger than the rest, one containing a calcified area and the other a prominent central accumulation of foam cells (Fig. 24A). A feature of note at this stage was the variable and sometimes prominent content of both elastic tissue and smooth muscle cells. The elastic tissue was especially prominent in the superficial aspect of many plaques and appeared to be "streaming" or splitting off from the internal elastic lamina at the margins of the plaque (Fig. 23C,D).

Eleven to eighteen weeks. Seventeen rabbits were examined at intervals from 11-18 weeks, and lesions were found in 39 blocks.

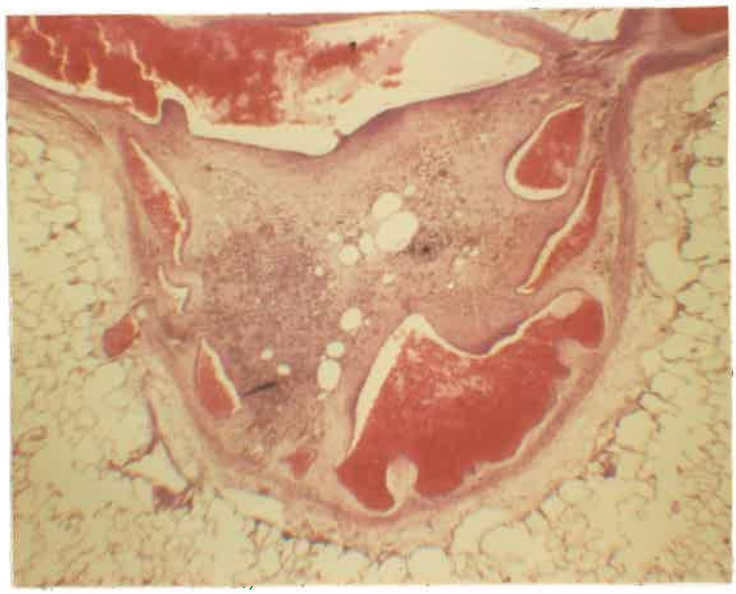
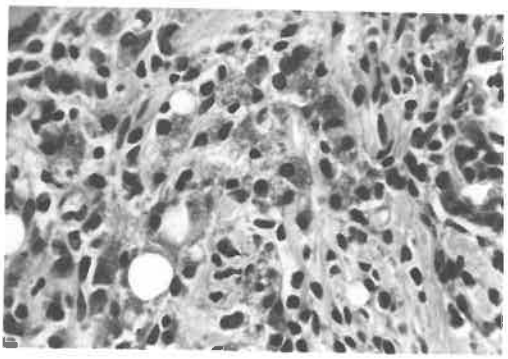
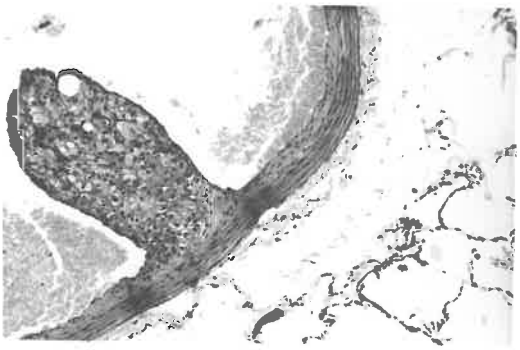


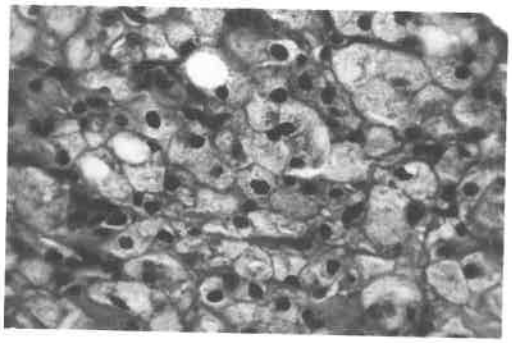
Fig. 21. 4 weeks after embolisation. The new vascular channels have a well defined layer of smooth muscle cells in their walls. H. & E. x 30.



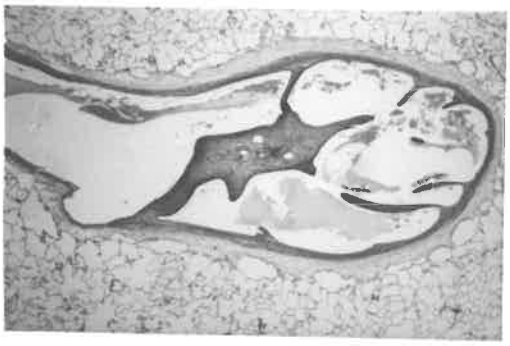
A. Foam cells in the lesion shown in Fig. 21. x 375.



B. x 85.

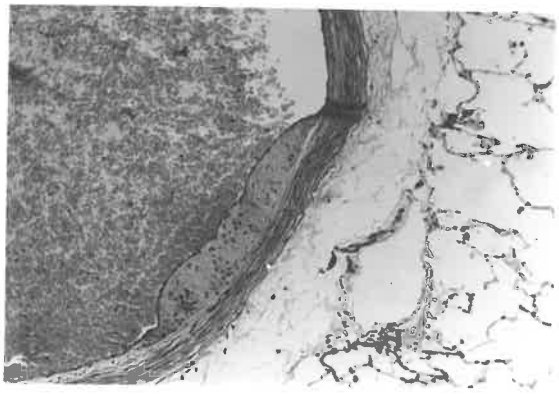


C. Foam cells in the lesion shown in Fig. 22B. x 375.

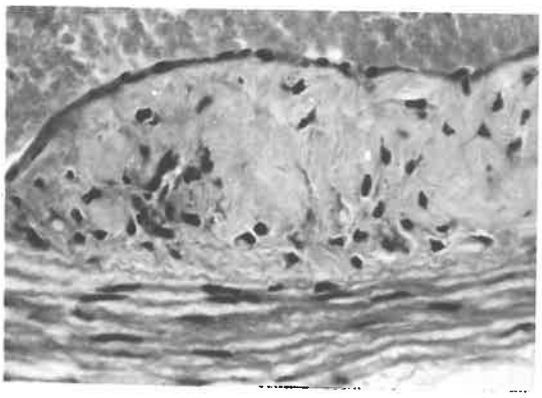


D. x 20.

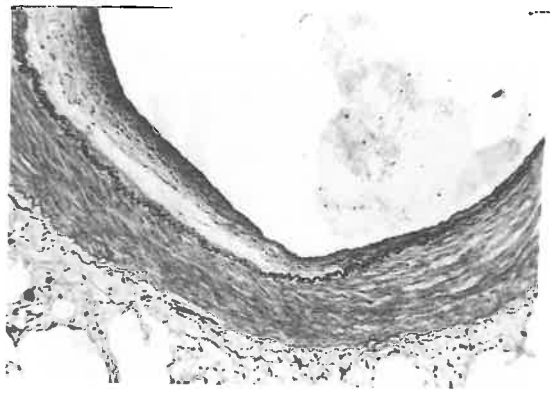
Fig. 22. Lesions at 4 weeks resulting from the organisation of plasma thrombi. H. & E.



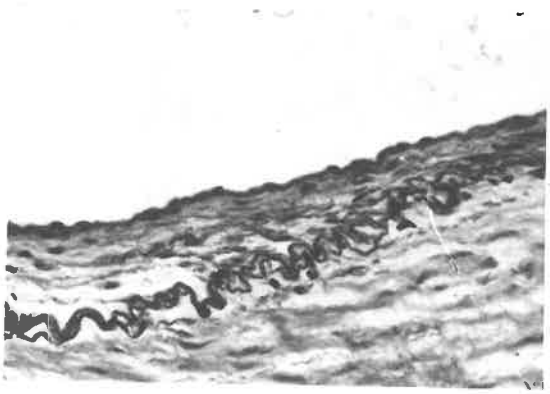
A. At 6 weeks. H. & E. x 85.



B. Edge of plaque shown in Fig. 23A. H. & E. x 375

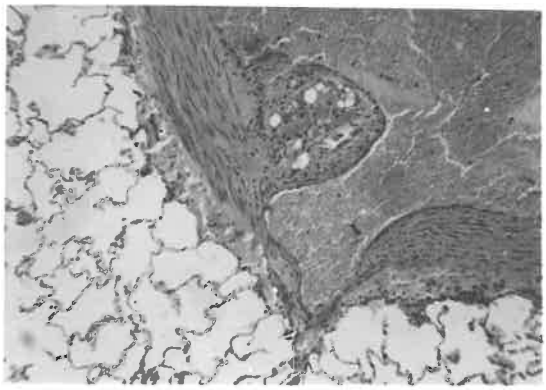


C. Gomori elastica. x 85.

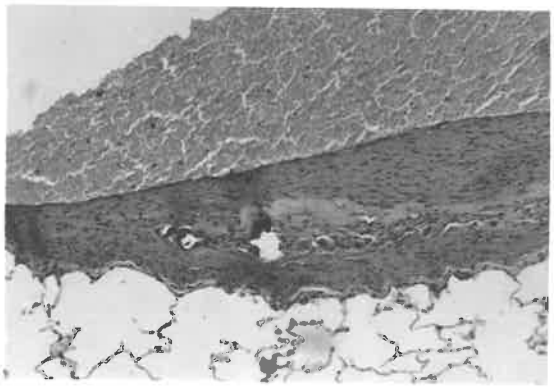


D. Edge of plaque shown in Fig. 23C. Gomori elastica. x 375.

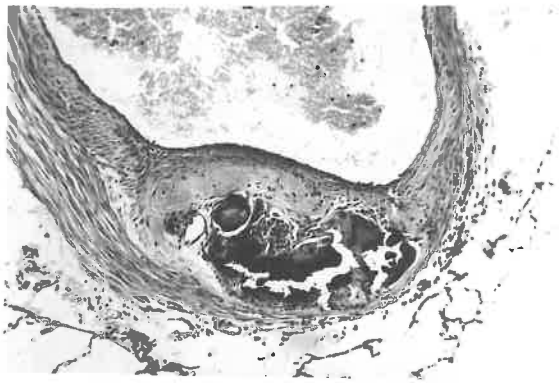
Fig. 23. Lesions at 6 - 8 weeks after embolisation.



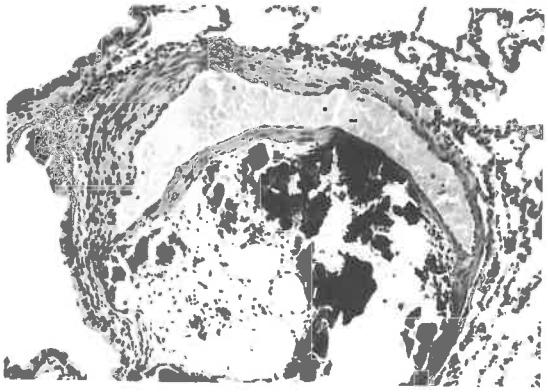
A. At 8 weeks.



B. 15 weeks.



C. 18 weeks.



D. 18 weeks.

Fig. 24. Lesions at 8 - 18 weeks. H. & E. x 85.

Some of the lesions were very small being discovered only by good fortune and the examination of a large number of sections. No lesions whatsoever were detected in three experiments (3, 5, 9). In 18 of the 39 blocks the lesions contained a variable but usually small accumulation of foam cells (Fig. 24B) and sometimes cystic lipid spaces were noted probably formed from the breakdown of foam-cells. The quantity of lipid within the plaques was significantly more prominent in two experiments and in one block the resulting lesion was polypoid in appearance and consisted almost entirely of foam cells as seen previously in Figure 22B. Seven blocks from three experiments (1, 8, 17) showed lesions containing calcified areas (Fig. 24C, D). It is of interest to note that the average number of blocks per experiment showing lesions had declined from 6.9 in the first week, to 4.0 at 4 weeks, and only 2.3 in the period 11-18 weeks.

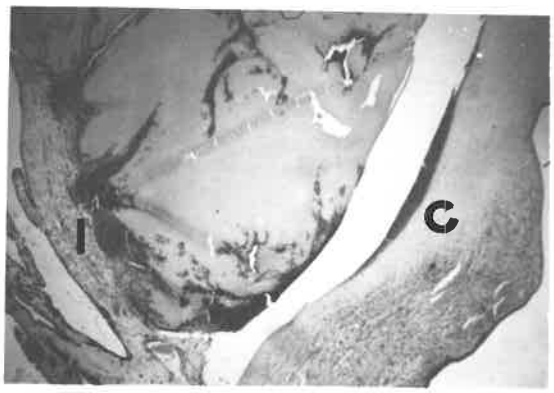
Figure 24C illustrates one final point. Medial atrophy was occasionally so marked in association with an intimal plaque and the vessel wall so thinned that an external bulging or aneurysmal dilatation of the vessel resulted.

The fate of plasma thrombi in the anterior eye chamber of the rabbit

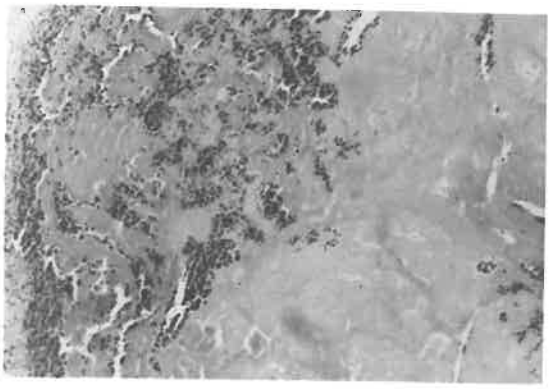
Observations were made at intervals from 2 days to 18 weeks in 39 rabbits. At operation, the thrombi had been pushed as far as possible into the irido-corneal angle. Microscopically the thrombi were close to, but usually not right in this angle, and were indenting the anterior surface of the iris.

Three days after insertion, a marked pseudo-eosinophil

polymorphonuclear leucocyte invasion of the thrombus was evident (Fig. 25A,B). This was only present when platelet aggregates were present in the thrombus, thus supporting the thesis that the leucocyte response is evoked by platelets within the thrombus, and not by fibrin alone. These leucocytes were seen emigrating from vessels in the limbus and in the iris. The pattern of organization followed closely that in the pulmonary arteries. Proliferating "endothelial" cells from both the posterior surface of the cornea (Fig. 25C) and the anterior surface of the iris (Fig. 25D) were observed on and after the third day, invading the thrombus, and covering its surface. These "endothelial" cells similarly appeared to possess a fibrinolytic property, and were clearly able to assume the morphology of fibroblasts. Macrophages, of uncertain origin, but possibly arising from emigrated circulating mononuclear cells were seen on the fourth day. Phagocytosis of degenerate polymorphonuclear leucocytes was observed, but definite platelet phagocytosis could not be demonstrated. Only occasional foam cells were seen. The paucity of foam cells, and the absence of demonstrable platelet phagocytosis may reflect the predominantly fibrinous nature of the thrombi inserted, the bulk of the head of each plasma thrombus having been used as pulmonary emboli. In accord with this possibility, of the 3 eye chamber experiments examined in the first week, only 2 provoked a significant polymorphonuclear response. Furthermore, it is not without interest that no increase in metachromatic material was observed in the stroma of the iris.



A. I = iris. C = cornea.



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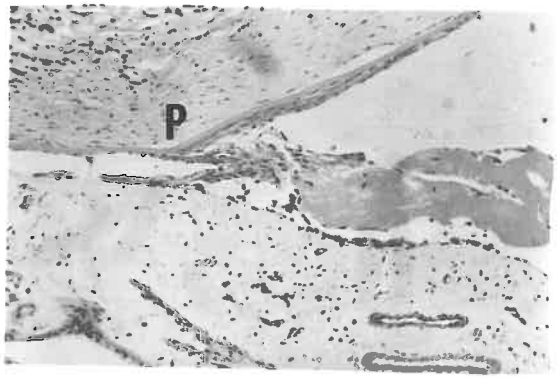


C. P = posterior aspect of cornea.

D. I = anterior surface of iris.

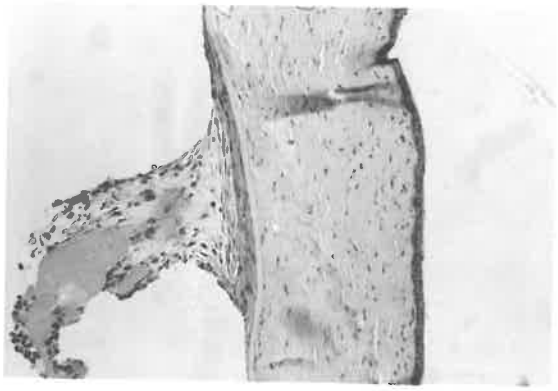
Fig. 25. Various aspects of the organisation of plasma thrombi in the anterior eye chamber of the rabbit. H. & E. x 85.

Two weeks after operation no residual thrombus or any resultant lesion could be found in one eye. In the other two eyes a tiny focus of thrombus remained, surrounded by cellular fibrous tissue attached to the posterior surface of the cornea (Fig. 26A, B). This, incidentally, was the latest stage at which residual thrombus could be seen in the pulmonary arteries.

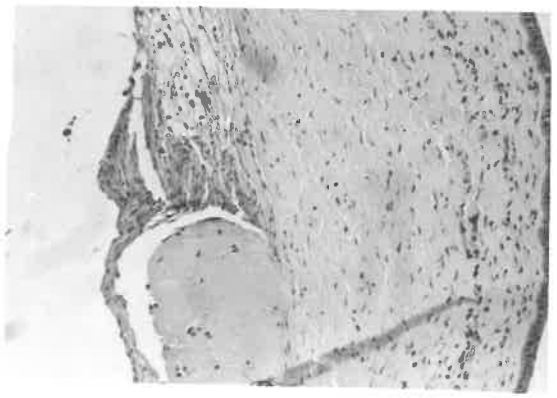
Between 4-8 weeks, only 5 of the 10 experiments showed lesions, and all that remained was a small flattened layer of cellular fibrous tissue attached to the posterior surface of the cornea (Fig. 26C). Foam cells were not present, but macrophages containing melanin pigment were present (Fig. 26D), this pigment presumably having arisen from the pigmented "endothelial" cells of the iris. After 8 weeks only one of the 18 experiments showed a lesion, similar to, though smaller than, that seen in the 4-8 week stage. Thus it would appear that the thrombi inserted into the rabbit's anterior eye chamber were removed more efficiently than those in the pulmonary artery, although the mechanisms of thrombus dissolution and organisation seemed to be the same in both sites. The differing and results could well relate to the lesser platelet component of the thrombi inserted into the anterior eye chamber.

The fate of whole-blood thrombi

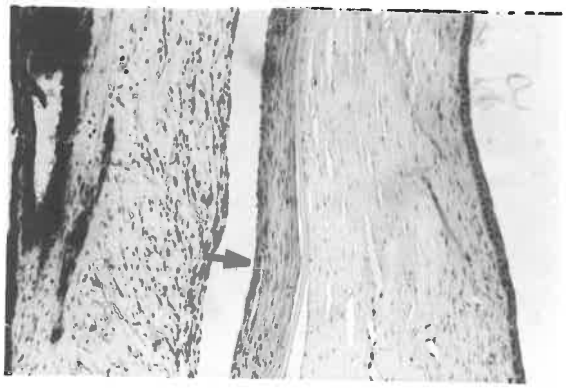
One week after operation emboli were found in 12 blocks derived from 3 experiments. Marked thrombolysis was evident at this stage, and in one experiment (72) although 8 large thrombi had been embolized, only one block revealed residual embolus;



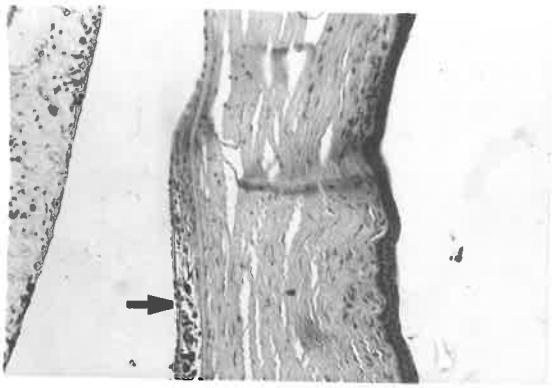
A.



B.



C.

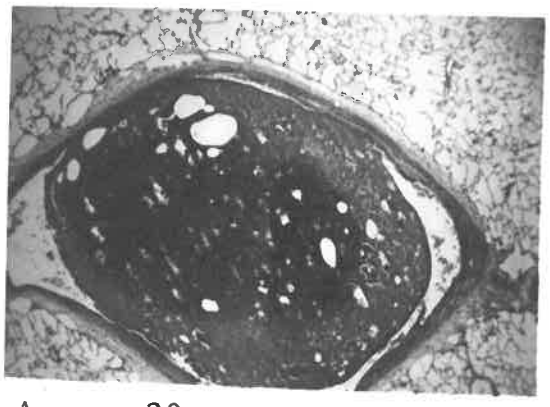


D.

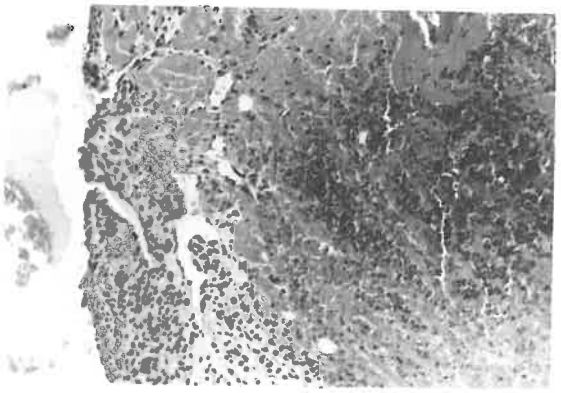
Fig. 26. Showing various stages in the organisation of plasma thrombi in the anterior eye chamber of the rabbit. H. & E. x 85.

the surface of this embolus was markedly irregular and excavated due to thrombolysis. At one week, the rate of organization of whole blood thrombi was comparable with that of plasma thrombi. Endothelialization was complete or almost so in most sections; new blood vessel formation was in evidence as was fibrosis. The vessel wall showed an increase in metachromatic material, the adventitial vessels were congested, and scattered lymphocytes were apparent in the adventitia. One impressive difference was the greater frequency and extent of the polymorphonuclear (pseudo-eosinophil) response to the whole blood emboli. This inflammatory response was present in all blocks containing emboli, and in some the infiltrate was massive (Fig. 27A, B, C). The author has interpreted this finding as reflecting the greater content of platelets in the whole-blood thrombi. Another obvious difference was the presence of greater numbers of foam cells in organizing whole-blood thrombi as compared with plasma thrombi, a finding also probably related to their greater platelet content. As would be expected, in the organizing whole-blood thrombi, there were numerous red cells in various stages of degeneration, including ghost forms, granular cells, and red cell fragments. Degenerate red cells were phagocytosed by macrophages and a few resulting haemosiderin-laden pigmented macrophages were evident.

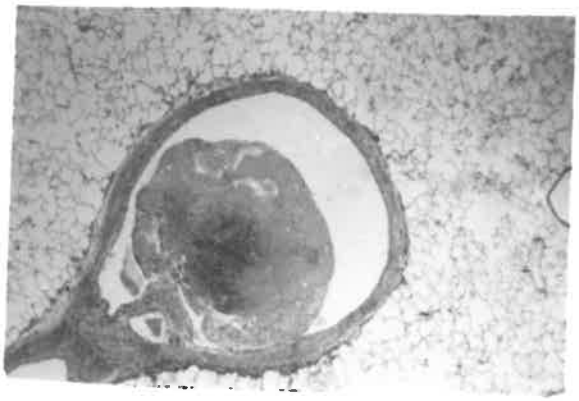
Two weeks after embolization 8 blocks derived from 3 experiments contained lesions. Whereas the organizing plasma thrombi at this stage showed a significant lipid content only



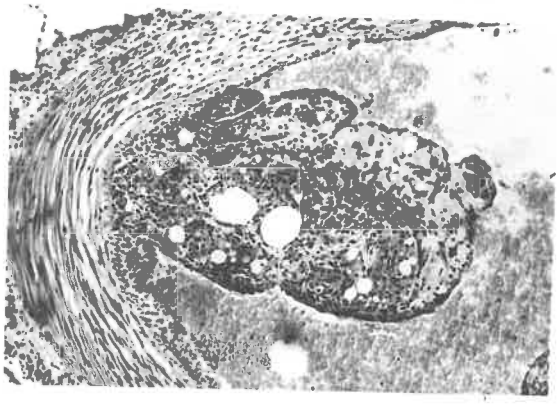
A. x 20.



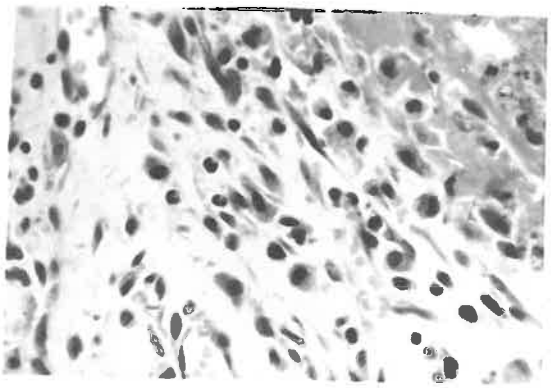
B. x 85.



C. x 20.



D. 2 weeks. x 85.



E. Part of the lesion shown in Fig. 27D. x 375.

Fig. 27. Various aspects of the organisation of whole blood thrombi. 1 - 2 weeks. H. & E.

infrequently, the organizing whole blood thrombi contained abundant lipid in 7 of 8 lesions examined, the remaining lesion showing extensive calcification. Calcification was present in lesions in each of the 3 animals in this group. One lesion consisted almost entirely of a mass of lipid-containing foam cells. At two weeks the lesions had diminished in size to occupy less than half the arterial lumen. Figure 27D shows a representative lesion, in which fibrosis, foam cells and pigmented macrophages (Fig. 27E) were present. In general the amount of haemosiderin pigment in these lesions, though variable, was small.

Organization was well-advanced by three weeks. Five of 7 lesions found contained copious lipid, and a further one was largely calcified. At this stage the rate of organization of whole-blood and plasma thrombi was comparable. At 4 weeks, the plaques were generally smaller, with the exception of one which contained a large amount of lipid.

Lesions were not detected in one experiment (62) at 6 weeks and in another (58) at 12 weeks. Calcification was seen on one occasion at 6 and 12 weeks respectively. Figures 28-30 illustrate the striking end result of the organization of autologous artificial whole-blood pulmonary thrombo-emboli, in rabbits 60, 57 and 59, the first at 6 weeks, and the other two at 12 weeks after embolization. Figure 29C shows the propensity of the vessel wall to bulge when the media has undergone atrophy. Cholesterol-like acicular clefts were seen (Figs. 29A and 30E) and the cholesterol content of these plaques

Fig. 28A. Macroscopic view of an eccentric lesion containing lipid.

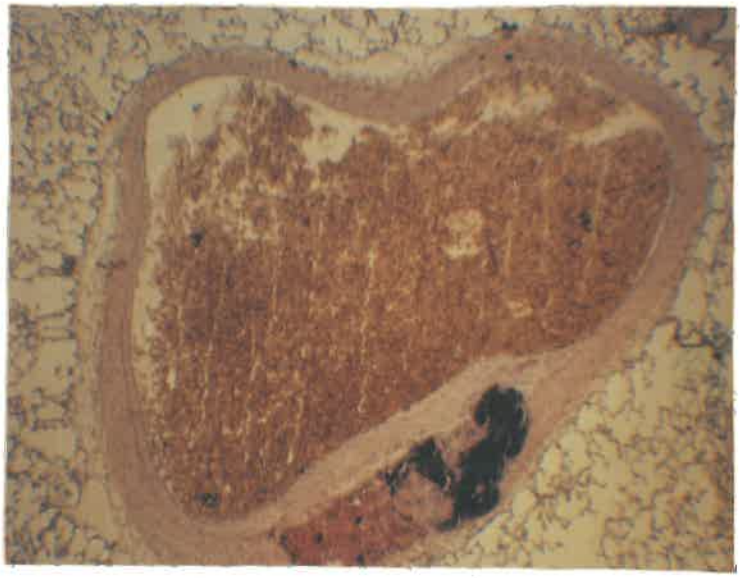


Fig. 28B. Same lesion as shown in Figure 28A. Scharlach R. x 30.

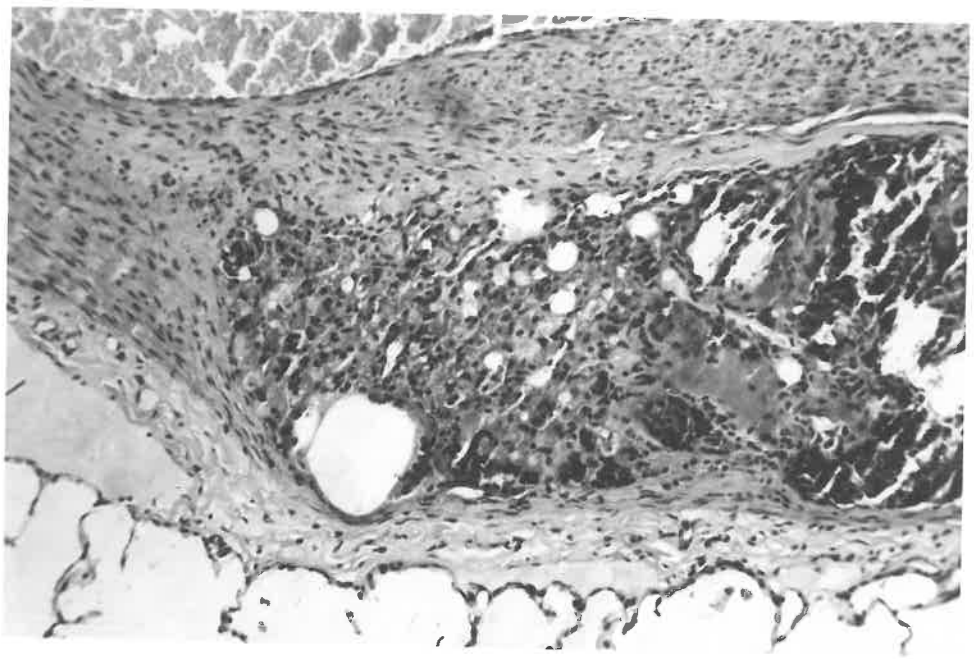
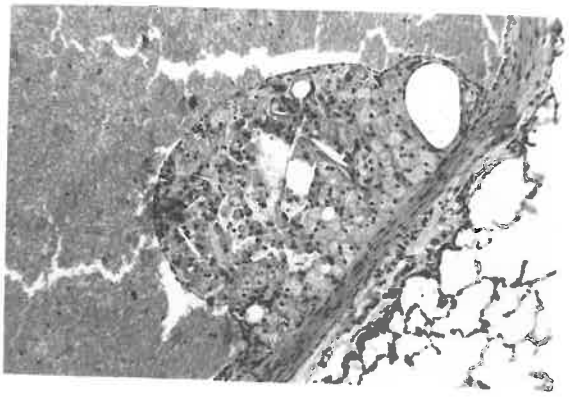
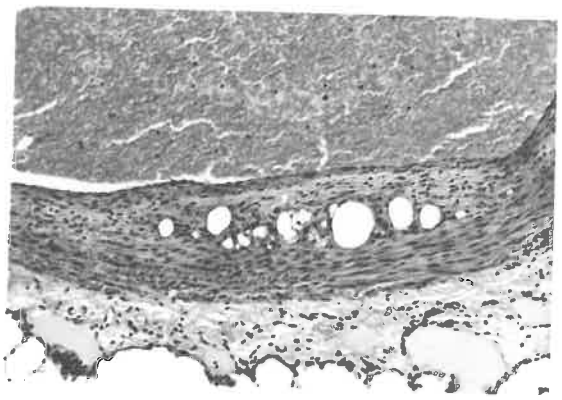


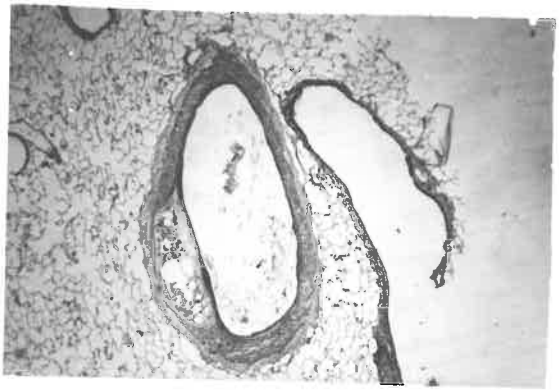
Fig. 28C. Foam cells and calcified material in the depths of the plaque. Note the smooth muscle cells superficially. H. & E. x 150.



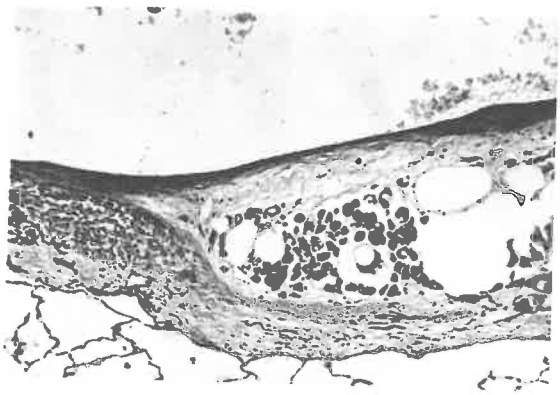
A. 6 weeks. x 85.



B. 6 weeks. x 85.

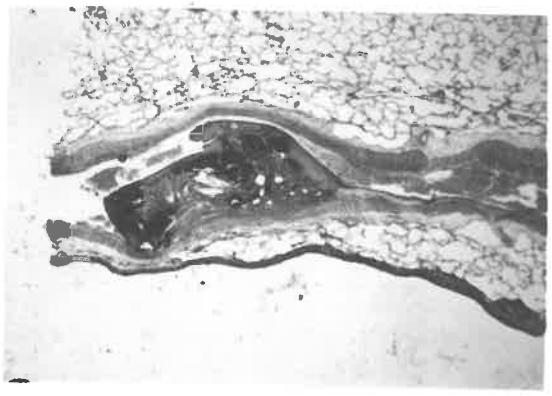


C. 12 weeks. x 20.

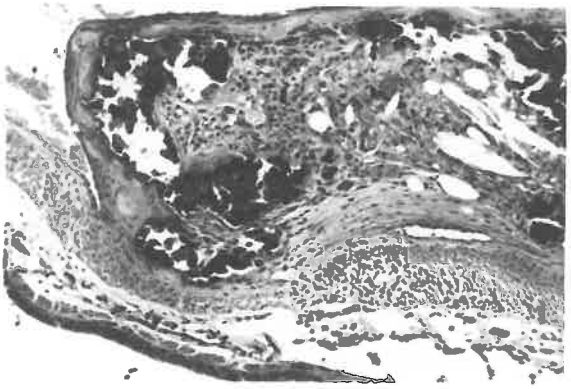


D. 12 weeks. Part of Figure 29C. x 85.

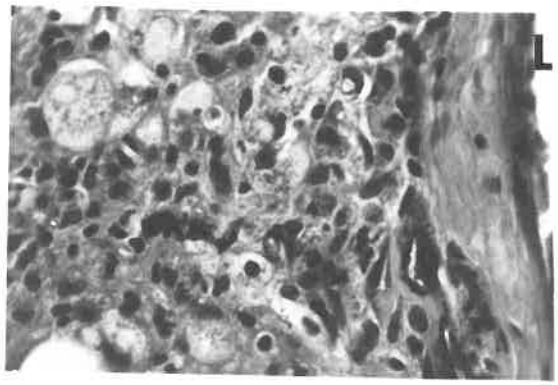
Fig. 29. Plaques at 6 - 12 weeks resulting from the organisation of whole-blood thrombi. H. & E.



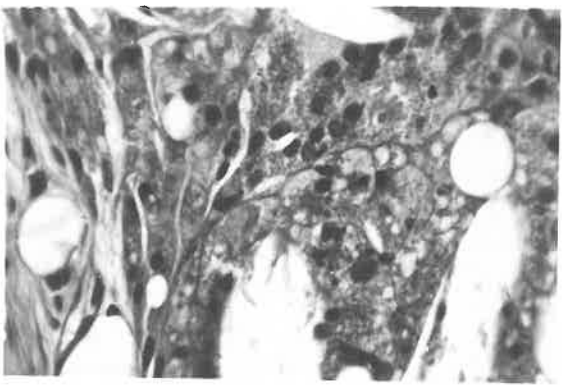
A. Overall. x 20.



B. Edge of plaque. x 90.



C. Superficial aspect.
L = lumen. x 395.



D. Deep aspect. x 395.

Fig. 30. Various aspects of a plaque at 12 weeks. H. & E.

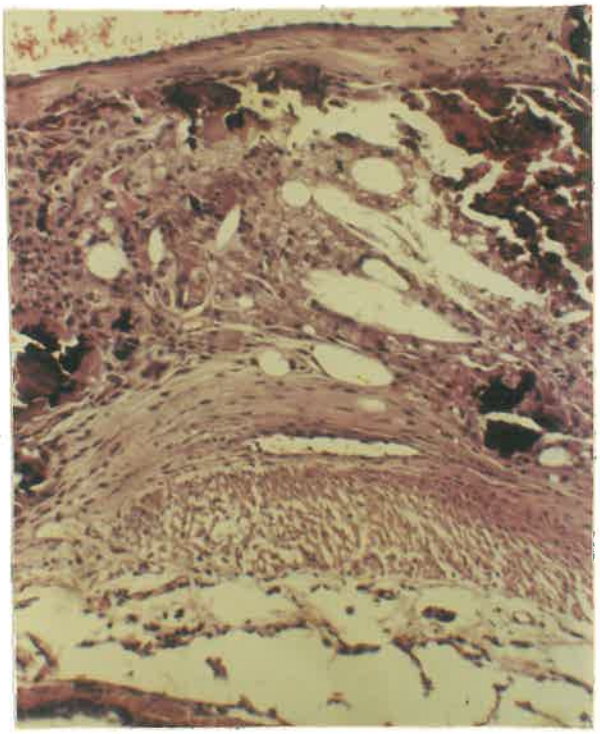


Fig. 30E. 12 weeks. The plaque contains foam cells, lipid cysts and obvious acicular clefts. The lumen of the vessel is at the top. H. & E. x 110.

was, in part, confirmed by the presence of birefrigent material.

Thus, the organization of a whole blood thrombus, in a normocholesterolaemic rabbit has resulted in the development of a plaque of significant proportion, a plaque with a dense fibrous cap and a centre containing foam cells, lipid cysts and cholesterol clefts, as shown in Figure 30E. The lesions resulting from whole blood emboli were larger than those produced by the organization of plasma thrombi, and clearly contained more lipid.

3. Discussion

In this study the changes occurring during the organization of autologous artificial plasma and whole-blood pulmonary thrombo-emboli in normo-cholesterolaemic rabbits has been described in detail. In addition, the organization of autologous plasma thrombi in the rabbit's anterior eye chamber has been studied. Observations were made at intervals ranging from 1½ hours to 18 weeks after operation. It was found that ultimately some of these thrombo-emboli evolved into complex fibrofatty plaques with many of the features of human "atheroma". These findings, in the author's opinion, add credence to the thrombogenic hypothesis originated by Rokitansky in 1852 and elaborated by Duguid in 1946.

A number of experimental models have been used in an attempt to evaluate this hypothesis, including the injection into the pulmonary arteries of fragmented human blood clot (Harrison, 1948), rabbit blood clot (Wartman, Jennings and Hudson, 1951; Barnard, 1954; Thomas, O'Neal and Lee, 1956), and fibrin clot

(Heard, 1952). Heptinstall (1957) utilized blood clot prepared from hypercholesterolaemic rabbits. In all cases the resulting pulmonary artery lesions were essentially fibrous intimal thickenings with little or no lipid. McLetchie (1952) was the first to show that fibrofatty intimal lesions might result from the organization of pulmonary artery thrombi. These he produced in rabbits by the repeated intravenous injections of Russell's viper venom. In 1962 Hand and Chandler studied the organization of artificial autologous whole-blood thrombi introduced into the pulmonary arteries of the rabbit. They found that the organization of these thrombo-emboli resulted in the development of fibrofatty intimal plaques rich in lipid-bearing foam cells which they suggested were derived from the phagocytosis of platelets. Their article, however, was poorly illustrated and the lesions depicted did not show the features typically seen in human atheromatous plaques. In this study, many facets of their work have been confirmed, but the observations have been extended and include studies on plasma thrombi, as distinct from whole-blood thrombi, introduced into the pulmonary arteries and also into an extravascular site, the rabbit's anterior eye chamber.

The earliest cellular response both in the pulmonary arteries and in the anterior eye chambers was a polymorphonuclear leucocytic infiltration of the thrombus. This was seen as early as $1\frac{1}{2}$ hours after embolization and by 48 hours was very prominent. Concomitant with this accumulation of leucocytes, the thrombus assumed an increasingly spongy appearance, with the development

of clear spaces surrounding the polymorphonuclear leucocytes. These changes probably reflect the fibrinolytic property of leucocytes, a property which has already been established in other situations (Gans, 1964; Riddle and Barnhart, 1964). It is uncertain whether degranulation of the polymorphonuclear leucocytes with release of lysosomal enzymes has contributed significantly to the process of thrombolysis, because apparently intact leucocytes were seen within these clear spaces, indicating that the intact cell can induce fibrinolysis. Using an in vitro technique to assess fibrinolytic activity, Gans (1964) concluded that autolysis of relatively few leucocytes will produce a rapid breakdown of considerable amounts of fibrin, and that the lysis resulted from both direct proteolytic activity and from plasminogen activator activity. Pertinent to these remarks, Dible (1958) noted that one stage of the process of " ... canalization seems to be the disappearance of the looser component part of the thrombus structure, either by autolysis or by leucocytic action ... ". Alternatively, as suggested by Riddle and Barnhart (1964) in their ultrastructural study of the inflammatory reaction in skin wounds, leucocytes may phagocytose fibrin which is then destroyed intracellularly. In these studies, however, no convincing evidence of fibrin phagocytosis was seen with the light microscope; however, intracellular fibrin was observed with the electron microscope, the results of which are presented in experiment 3 of this study.

A noteworthy finding, both for whole-blood and plasma thrombo-emboli, was the degree of variation of the polymorphonuclear

leucocyte response. It was greatest in those thrombi containing copious platelet aggregates, and was absent or minimal when platelets were sparse or absent. This finding suggests that platelets release a leucotactic factor, and it is this, rather than the presence of fibrin or red cells, which determines the polymorphonuclear leucocyte response. Hirsch (1960) demonstrated that platelets impart bactericidal properties to rabbit blood serum. Ryan and Hurley (1966) logically, but theoretically extended this activity of platelets to include chemotaxis to polymorphs.

Phagocytosis and the origin of foam cells

A second population of cells entered the thrombus shortly after the leucocyte response. This mononuclear cell infiltration included circulating lymphocytes and monocytes, and a third cell type, with a nucleus larger than that of a monocyte, a looser chromatin network, and one or two prominent nucleoli. The origin of these cells was uncertain, and they could have been derived from either monocytes or lymphocytes. This suggestion receives support from the experiment of Gillman and Wright (1966) which provided autoradiographic evidence of in vivo trnasformation of blood mononucleus into macrophages and other cell forms. These large mononuclear cells increased in number, and assuming phagocytic properties, ultimately evolved into macrophages. This increase in number was not associated with any appreciable mitotic activity. Hand and Chandler (1962) concluded that the lipid-containing macrophages, or lipophages

as they called them, were derived from blood monocytes, but in view of the difficulties inherent in determining the origins of a mixed population of mononuclear cells, judgement is reserved on this important point.

Phagocytosis was first apparent at 48 hours, and subsequently increased in intensity. Mononuclear cells were seen to engulf both nuclear and other debris from degenerating polymorphonuclear leucocytes, red cell fragments, and platelets. After ingestion, the platelets became greatly swollen and vacuolated, making their intracellular identification difficult in most instances. By the fourth day, scattered foam cells containing lipid were in evidence. Some of these cells were almost certainly derived by the mononuclear phagocytosis of platelets, and transitional stages between early phagocytosis and the established foam cell could be seen, thus confirming the observations of Hand and Chandler (1962).

However, not all of the foam cell lipid is necessarily derived from platelets, although platelet lipid, which accounts for up to 25% of the dry weight of platelets (Woodside et al., 1964) is probably the most important source. Another source is the leucocyte, which is alleged to be even more active than the platelet in lipid synthesis (Kidson, 1961). Plasma lipid, contained or trapped within the thrombus could theoretically be a third source, but in normocholesterolaemic rabbits with very low cholesterol levels of only 20-80 mg./100 ml. this is unlikely to make a significant contribution. Red cells were suggested by Duguid (1946) as a

possible source of lipid in organizing thrombi. For two reasons, this is unlikely. Firstly, extensive foam cell formation was observed using plasma (red-cell free) thrombo-emboli; and secondly, lipid only accounts for approximately 1% of the dry weight of a red cell (Barkhan, Silver and O'Keefe, 1961). Finally, one should consider the possibility of lipid synthesis within the macrophages during phagocytosis, but this is probably not a significant contributing factor.

Just as the intensity of the polymorphonuclear response appeared to relate to the number of platelets within the thrombo-embolus, so also did the extent of the foam cell reaction, a finding which supports the conclusion that platelets and possibly also white cells are vital to the genesis of foam cells in the organization of thrombi in this experimental situation.

Endothelialization and vascularization

Endothelialization was in evidence 3 days after embolization, when a layer of flattened cells was seen, beginning to spread over the free surface of the thrombus. This cell-layer appeared continuous with the vascular endothelium at the margins of the thrombus where proliferation with some mitotic activity was noted. Growth of the vascular endothelium appeared to be the principle mechanism of endothelialization. It is possible, however, that another mechanism might contribute to the process of endothelialization. Flattened cells were observed on the free surface at points removed from the advancing margins of

endothelium, and transitional forms, intermediate between circulating mononuclears and flattened endothelial-like cells were seen, suggesting that an undifferentiated mononuclear cell might be capable of an endothelial transformation. This alternative receives some support from the study of Halpert and colleagues (1966) who suspended a hub in the lumen of a vessel in such a way that endothelialization arising from the vascular endothelium was impossible. They concluded that the endothelium covering the hub was, therefore, derived from circulating multi-potential cells of uncertain origin. In an earlier study, Ghani and Tibbs (1962) had also suggested that endothelial cells could be derived from circulating mononuclear cells.

After the third day spindle cells began to appear within the thrombus; because of the presence of transitional forms, it appeared that some spindle cells were derived from circulating mononuclear cells, but the main source of these fibroblast-like cells were the proliferating endothelial cells on the surface and at the base of the thrombus. The ability of endothelial cells to change into fibroblasts and other cells has been extensively documented by Altschul (1950). This "endothelial" invasion was accompanied by an interstitial metachromatic basophilia and the formation of fine collagen fibres, indicating the fibroblastic role these cells had assumed. An association between fibroblastic activity and interstitial metachromasia has been described in granulation tissue by Taylor and Saunders (1957), and Lovell et al. (1966). Elastic-

staining fibres made their appearance at this time, but how these were produced remains uncertain. Around some of the invading "endothelial" cells, clear zones were noted, suggesting that these, like the polymorphonuclear leucocytes, might also posses fibrinolytic properties. This is not surprising in view of the well documented fibrinolytic properties of vascular endothelium (Warren, 1963, 1964, 1965). Moreover, the "neo-intima" or new endothelial cells lining aortic grafts also possess fibrinolytic activity (Warren and Brock, 1964).

Vascularization of the organizing thrombi commenced at 3-4 days, and two mechanisms appeared to be involved; an ingrowth of "endothelial" cells lining spaces and crevices within the thrombus; and the canalization of solid "endothelial" buds which were seen to grow into the thrombus. Many of the new vessels showed a communication with the vascular lumen at the margins of the organizing thrombus, but definite communication with the adventitial vasa vasorum was rarely observed, and then usually only at points of branching, or when the media was markedly thinned. These observations are in complete agreement with those of Dible (1958) who concluded that "... canalization of arterial thrombi takes place within the internal elastic lamina ...".

Smooth muscle cells

Smooth muscle-like cells were observed in the organizing thrombi in association with the development of new vascular channels and at the surface of plaques. Their recognition was not easy,

differentiation from fibroblasts being a major problem. Their probable source is the multipotential endothelial cell, although the possibility that they might arise from undifferentiated circulating mononuclear cells could not be excluded. On the other hand, there was no evidence whatsoever that medial smooth muscle cells, which have been incriminated as the source of smooth muscle in organizing thrombi in animals and man (Malyschew, 1929) have played any part in the origin of the smooth muscle cells in the plaques. Moreover, in human arteries and veins, the author has failed to find any evidence of medial smooth muscle cell participation in the process of organization. Finally, smooth muscle cells did not appear to contribute to the formation of foam cells.

Medial and adventitial changes

These were threefold; firstly, medial thinning in the later stages of organization, ultimately, in some instances, progressing to aneurysmal bulging of the wall; secondly, an interstitial basophilic oedema of the media, which was metachromatic and alcian blue positive. This change was prominent at 48 hours, when some degeneration of muscle and elastic fibres was also noted, but by two weeks was, with one exception, no longer visible. Thirdly, changes were noted in the adventitia, including a polymorphonuclear leucocytic response, together with a slight to moderate lymphocytic infiltration, and marked congestion and dilatation of the vasa vasorum, particularly when the emboli were occlusive. These vessels might serve as collateral channels in such a situation (Morgan, 1956).

Medial thinning might feasibly result from ischaemia of the arterial wall. Other possibilities include a splinting effect, with disuse atrophy of muscle, or a pressure effect of the plaque itself, as suggested by Morgan (1956). All possibilities have something to commend them, though their relative importance remains uncertain.

The most striking change produced in the vessel wall at, and adjacent to, the point of impaction of a thrombo-embolus, was an obvious increase in the amount of intercellular metachromatic material associated with a wide separation of the medial muscle and elastic fibres. This change might have resulted from an impaired trans-endothelial nutrition. On the other hand, both platelets and leucocytes contain a number of substances including serotonin, histamine, adrenaline, adenosine triphosphate and lysosomal enzymes, which might increase permeability, or produce vascular injury (Hughes and Tonks, 1962; Mustard et al., 1964); the latter could also contribute to medial thinning. These possibilities clearly need evaluation.

The fate of anterior eye chamber implants

Most facets of the organization of plasma thrombi in this extravascular site were comparable with the organization of plasma pulmonary thrombo-emboli, but there were two notable differences. Firstly, the eye chamber implants, which generally contained less platelets, did not provoke so marked a polymorphonuclear response, and the development of foam cells was less conspicuous. Secondly, the implants did not progress to the late stage fibrofatty plaque seen

in the pulmonary arteries; the observed differences could well relate to the paucity of platelets in the chamber implants. This study has shown, however, that the phagocytosis of platelets in an extravascular site will results in the development of lipid containing foam cells, suggesting that the development of an atherosclerotic plaque may not be dependent on any process in the wall of an artery. Further experiments with thrombi containing abundant platelets will be necessary to evaluate this suggestion.

A comparison of plasma and whole-blood thrombo-emboli

Many aspects of the organization of plasma and whole-blood pulmonary thrombo-emboli were comparable, including the rate of endothelialization, new vessel formation, fibrosis, and changes in the vessel wall. However, the frequency and extent of the polymorphonuclear leucocyte response in the whole-blood emboli was appreciably greater than in the plasma thrombo-emboli. A second difference was the presence of an obviously larger number of foam cells in the organizing whole-blood thrombi. Both differences are probably related to the greater initial platelet content of the whole-blood thrombi embolized, a finding which was confirmed on serial section of a number of control plasma and whole-blood thrombi. The increased foam cell content of the organizing whole-blood thrombo-emboli could be further explained on the basis of additional lipid derived from the phagocytosis of red cell fragments, and a larger amount of polymorphonuclear debris.

End-stage lesions

The end result of the organization of autologous artificial thrombo-emboli, particularly those derived from whole-blood was, in many instances, an eccentric complex fibrofatty plaque, with a dense collagenous cap overlying a central core of lipid (Fig. 30E). Both intra- and extracellular lipid was present, together with acicular cholesterol clefts and lipid cysts. The cholesterol content of at least some of these plaques was confirmed by the presence of birefringent material. Vascularization and calcification were sometimes prominent. Thinning of the media was not uncommon and occasionally an aneurysmal bulging of the media resulted. Rupture of the lipid core into the adventitia was noted once, but no evidence of plaque ulceration or surface thrombosis was seen. While the eye chamber implants of plasma thrombi resulted in fibrofatty lesions, they did not evolve into such complex lesions as did the pulmonary thrombo-emboli, probably due to their lower platelet content.

It is of importance that these pulmonary artery plaques have developed in the absence of any cholesterol-feeding. Their resemblance to the naturally occurring lesions of man is impressive. Thus, it would appear that the experimental model employed in this series of experiments has succeeded in the production of a convincing counterpart to the human lesion, a finding which must help to dispel the arguments raised by a number of workers, including Constantinides (1965) that the thrombogenic hypothesis "... cannot, by itself, explain

the development of fatty gruel within the center of pearly plaques. A thrombus just does not contain the tremendous quantities of cholesterol and other lipids we find in the gruel". Constantinides continued - "Finally, a serious weakness of the thrombogenic theory is the fact that it has been impossible so far to cause the transformation of experimental arterial thrombi into typical pearly plaques with capsule, gruel, capillarization, calcification, and the rest of their usual features and complications". Just this has been done.

Experiment 2: The influence of hypercholesterolaemia on the organisation and fate of autologous pulmonary thromboemboli

1. Materials and Methods

Sixty-one rabbits were given a cholesterol-enriched diet, which was commenced 3 weeks prior to operation. This was prepared by dissolving 100 g. of cholesterol (British Drug Houses Ltd., England) in 1100 ml. of anaesthetic ether, and adding to this solution 900 ml. of peanut oil. At least 48 hours before use, 120 ml. aliquots of this cholesterol-ether-oil mixture were homogenized with 900 g. of a mashed form of rabbit pellets, so that each 100 g. of mash contained 0.67 g. of cholesterol. The rabbits were fed this diet ad lib. All rabbits were maintained on this diet until killed or for a maximum period of 7 weeks. Thus, in the group killed 8 weeks postoperatively, cholesterol feeding was stopped 4 weeks after operation, the standard pellet diet being given thereafter. In this study, 22 rabbits served as controls and were killed at intervals

TABLE 5

TOTAL SERUM CHOLESTEROL LEVELS AT VARYING TIMES AFTER THE COMMENCEMENT OF CHOLESTEROL FEEDING. THE ANIMALS RECEIVING PLASMA THROMBO-EMBOLI ARE GROUPED ACCORDING TO TIME AFTER OPERATION.

PLASMA THROMBO EMBOLI										
		Time after cholesterol diet commenced (weeks)								
Weeks post-operative	Exp. number	0	1	2	3 Operation	4	5	6	7 Diet discontinued	11
1	31/CH	35	240	360	890	1300				
	32/CH	50	410	600	930	890				
	33/CH	50	340	460	930	1050				
	34/CH	50	390	600	1040	934				
2	21/CH	70	500	770	1350	-	1500			
	25/CH	20	195	140	220	-	492			
	29/CH	60	210	520	900	-	1450			
	30/CH	35	80	225	570	-	1000			
3	10/CH	40	150	450	420	-	-	780		
	14/CH	80	230	980	1450	-	-	1275		
	15/CH	30	240	415	540	-	-	920		
	19/CH	30	40	165	550	-	-	660		
4	6/CH	50	800	1060	1450	-	-	-	2800	
	8/CH	60	520	490	385	-	-	-	580	
	16/CH	45	250	610	840	-	-	-	1000	
	17/CH	45	55	260	300	-	-	-	730	
8	1/CH	40	115	220	280	-	-	-	886	280
	2/CH	50	425	365	500	-	-	-	970	720
	4/CH	45	240	410	570	-	-	-	740	210
	7/CH	60	260	350	460	-	-	-	490	100
	9/CH	40	430	440	460	-	-	-	540	300
	12/CH	50	125	640	770	-	-	-	1415	225

TABLE 6

TOTAL SERUM CHOLESTEROL LEVELS AT VARYING TIMES AFTER THE COMMENCEMENT OF CHOLESTEROL FEEDING. THE ANIMALS RECEIVING WHOLE BLOOD THROMBO-EMBOLI ARE GROUPED ACCORDING TO TIME AFTER OPERATION.

WHOLE BLOOD THROMBO EMBOLI										
		Time after cholesterol diet commenced (weeks)								
Weeks post-operative	Exp. number	0	1	2	3 Operation	4	5	6	7 Diet discontinued	11
1	59/CH	35	550	840	920	1300				
	60/CH	35	360	845	1125	1300				
	61/CH	60	500	840	722	1690				
2	56/CH	20	280	630	1000	-	1640			
	57/CH	35	560	970	1650	-	2075			
	58/CH	30	150	520	584	-	985			
3	40/CH	50	600	930	788	-	-	-		
	41/CH	30	290	600	920	-	-	935		
	55/CH	25	470	720	588	-	-	1610		
4	35/CH	50	640	880	1125	-	-	-	1725	
	38/CH	60	640	672	1150	-	-	-	1550	
	53/CH	30	560	940	1400	-	-	-	1800	
	54/CH	40	540	880	768	-	-	-	1250	
8	27/CH	15	360	680	1000	-	-	-	2780	1925
	36/CH	40	200	292	230	-	-	-	425	135
	37/CH	36	720	788	780	-	-	-	1625	275
	39/CH	80	990	1100	1665	-	-	-	1945	400

TABLE 7

TOTAL SERUM CHOLESTEROL LEVELS AT VARYING TIMES AFTER THE COMMENCEMENT OF CHOLESTEROL FEEDING. THE CONTROL ANIMALS ARE DIVIDED INTO GROUPS COMPARABLE WITH THE TIME AFTER OPERATION IN THE TWO EXPERIMENTAL CATEGORIES.

CONTROLS - NOT OPERATED

Comparable time post-operative	Exp. number	Time after cholesterol-diet commenced (weeks) 0	1	2	3	4	5	6	7 Diet discontinued	11
0	20/CH	25	200	650	600					
	22/CH	25	240	460	450					
1 week	28/CH	20	210	480	650	1000				
	42/CH	40	388	570	730	790				
	45/CH	65	500	950	1000	1100				
	51/CH	40	600	755	1000	950				
2 weeks	24/CH	50	285	730	560	-	1020			
	26/CH	30	220	290	450	-	546			
	49/CH	58	845	1360	1700	-	2500			
	50/CH	44	500	960	1350	-	2100			
3 weeks	18/CH	35	310	260	460	-	-	650		
	23/CH	25	265	340	570	-	-	720		
	48/CH	43	323	660	850	-	-	900		
	52/CH	82	756	960	1350	-	-	1615		
4 weeks	11/CH	35	500	1000	1450	-	-	1500	1500	
	13/CH	25	285	470	700	-	-	-	680	
	44/CH	40	410	640	990	-	-	-	1730	
	46/CH	45	408	850	940	-	-	-	1575	
	47/CH	45	470	790	980	-	-	-	1100	
8 weeks	3/CH	50	135	190	340	-	-	-	560	102
	5/CH	50	300	540	500	-	-	-	970	230
	43/CH	40	640	570	800	-	-	-	1650	650

corresponding to 0, 1, 2, 3, 4 and 8 weeks postoperatively (Table 7). Of the remaining 39 rabbits, 22 had plasma and 17 had whole-blood pulmonary thrombo-emboli introduced, and these were killed at 1, 2, 3, 4 and 8 week intervals after operation (Tables 5 and 6).

The methods employed in the production of the artificial thrombi, the operative procedure, and the manner in which the lungs were prepared and examined were as described in experiment 1.

Total serum cholesterol

These levels were estimated serially on marginal ear-vein samples with a Technicon Auto-analyser employing a modification of the method described by Zlatkis, Zak and Boyle (1953).

2. Results

Total serum cholesterol

Tables 5 and 6 show the total serum cholesterol levels in the groups in which plasma and whole-blood thrombo-emboli were introduced. Table 7 shows similar data for the 22 non-operated or control animals which have been divided into groups corresponding to the times after embolization at which the operated animals were killed. It can be seen that the serum cholesterol level before cholesterol feeding was commenced ranged from 20 to 80 mg./100 ml., and that the levels in the three groups were comparable at different times after the commencement of cholesterol feeding.

Pulmonary artery lesions in cholesterol-fed rabbits

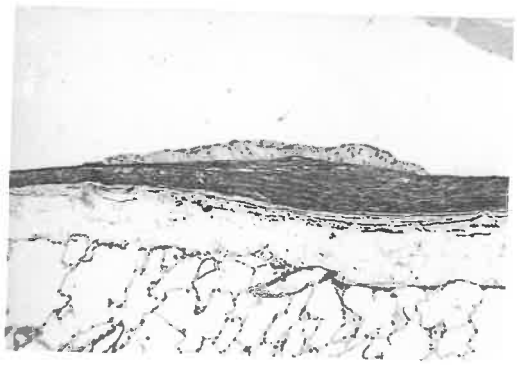
In order to establish a base line, and to determine the effects of cholesterol feeding alone,, 22 rabbits were killed at times

corresponding to those when observations were made on the embolized animals.

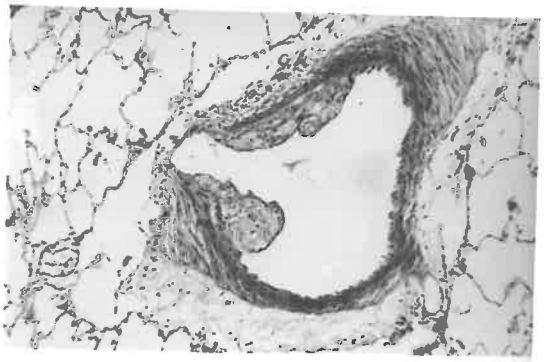
Two rabbits were killed three weeks after the commencement of the cholesterol-enriched diet - that is, at the time when emboli were introduced. In these, despite the fact that their terminal serum cholesterol levels were 600 and 450 mg/100 ml., detectable changes in the pulmonary arteries were minimal. Occasional isolated foam cells were seen free in the lumina of vessels, and in two blocks similar cells were found adhering to the endothelial lining at points of branching. Beneath one of these surface collections, cells in an intimal branch cushion, possibly smooth muscle cells, also contained lipid droplets. No lipid was detected in the endothelial cells.

During the subsequent weeks, changes consisting predominantly of the accumulation of subendothelial foam cells became more obvious, and in each of the groups, the extent of these lesions was greatest in those animals with the highest blood cholesterol levels. After 7 weeks of cholesterol-feeding subendothelial accumulations of foam cells were abundant in many arteries (Fig. 31A) and veins (Fig. 32). Numerous circulating foam cells were apparent at this stage, and the arterial lesions showed a striking predilection for points of branching (Fig. 31B).

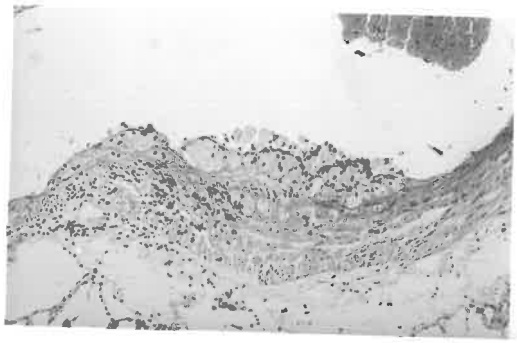
Lesions were still abundant in the group examined 4 weeks after the cessation of cholesterol-feeding. Fibrosis was minimal in all but one of the lesions seen (Fig. 33) which was the most advanced plaque resulting from cholesterol-feeding alone. Foam cells, adhering to



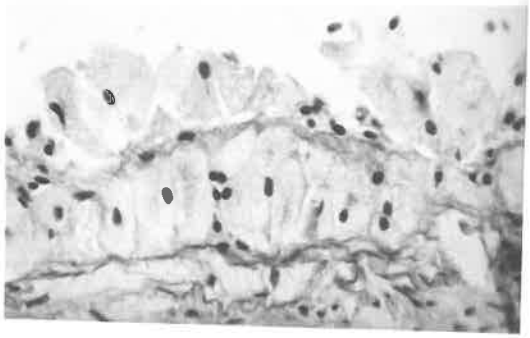
A. Gomori elastic. x 85.



B. H. & E. x 85.



C. H. & E. x 85.



D. Higher magnification of Figure 31C. H. & E. x 375.

Fig. 31. Lesions in the pulmonary arteries produced by cholesterol feeding.

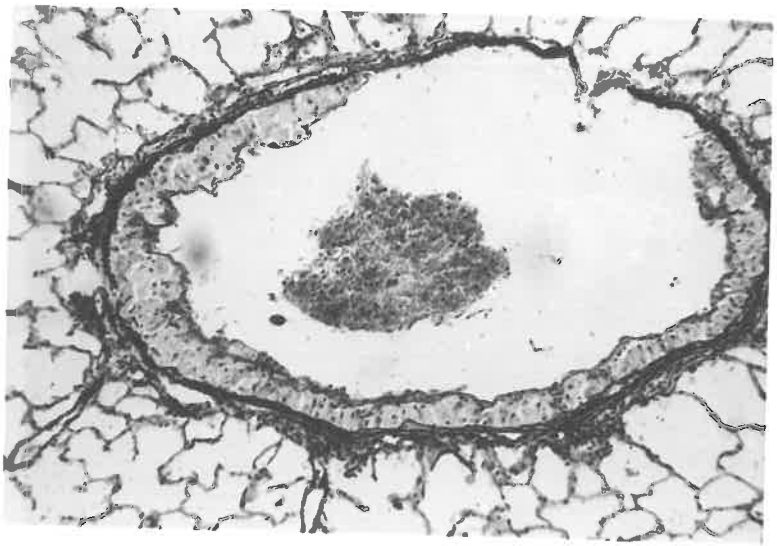


Fig. 32. Gomori elastic. x 30.

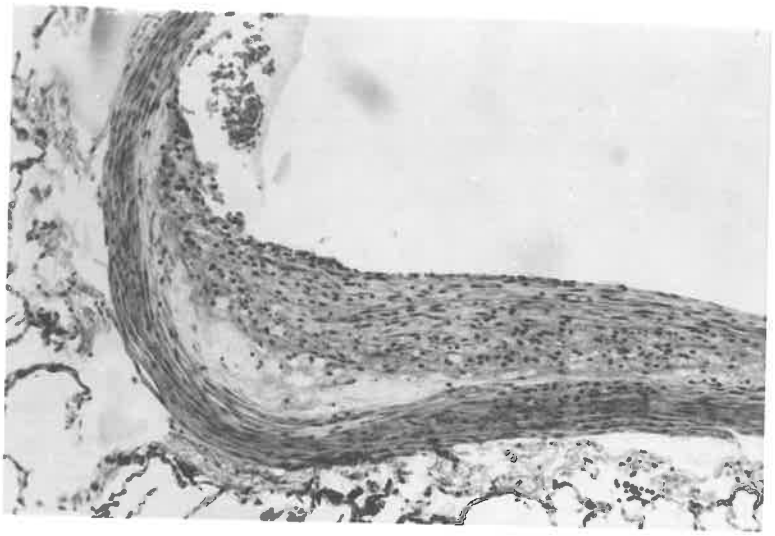


Fig. 33. H. & E. x 120.

Lesions produced by cholesterol feeding.

the endothelium were still in evidence.

As an origin for these foam-cell lesions, Rennie and Duguid (1953) suggested the endothelialization of foam cell collections adherent to the arterial surface. Our findings support this view. Figures 31C and 31D show several foam cells attached to the arterial wall and becoming endothelialized. More advanced lesions appeared to develop by the successive adherence and endothelialization of further foam cells (Fig. 31D). Later, the endothelial cells appeared to assume the morphology and activity of fibroblasts, resulting in fibrosis as seen in Fig. 33. It should be stressed that the lesions produced by only 7 weeks of cholesterol feeding were generally little more than subendothelial foam cell accumulations. Thus, there was no likelihood that the lesions resulting from cholesterol feeding alone would be confused with those resulting from the organization of thrombo-emboli. Additionally, no thrombi were seen in these rabbits.

Fate of plasma thrombo-emboli

At 1 week after embolization organizing plasma thrombo-emboli in hypercholesterolaemic rabbits shared many features in common with organizing plasma thrombo-emboli in those animals on a normal diet. The vessel wall showed a variable and sometimes marked accumulation of metachromatic material, the adventitial changes of congestion and lymphocytic infiltration were present, endothelialization of the surface of the thrombi was well advanced, or complete, and most of the thrombo-emboli were still virtually occlusive. The process of

thrombolysis and the mode of organization appeared to be similar in both groups, with the presence of "endothelial" cells, pseudo-eosinophilic polymorphonuclear leucocytes, and a variety of cells derived from circulating mononuclear cells. However, several differences were apparent. In the hypercholesterolaemic group there was an obvious difference in both the amount and frequency of calcification. In rabbits on a normal diet, only 5 of 33 blocks containing thrombo-emboli at one week showed microscopic calcification, while in the hypercholesterolaemic animals at one week calcification was seen in 12 of 27 blocks. This difference between the groups was found to persist through the later stages of the experiment. A second and important difference was the presence, even at 1 week, of a significantly greater number of lipid-containing cells within the organizing emboli (Fig. 34A). As might be expected, a small number of circulating foam cells were seen adhering to the surface of these organizing thrombi. These were subsequently incorporated.

In the four experiments comprising the group examined 2 weeks after operation, thrombi in various phases of organization were seen in 37 blocks, and in 18 of these a considerable amount of residual thrombus was present. When compared with this stage of organization in normocholesterolaemic rabbits, it was obvious that the amount of thrombus remaining in the hypercholesterolaemic group far exceeded that seen in the rabbits on a normal diet,

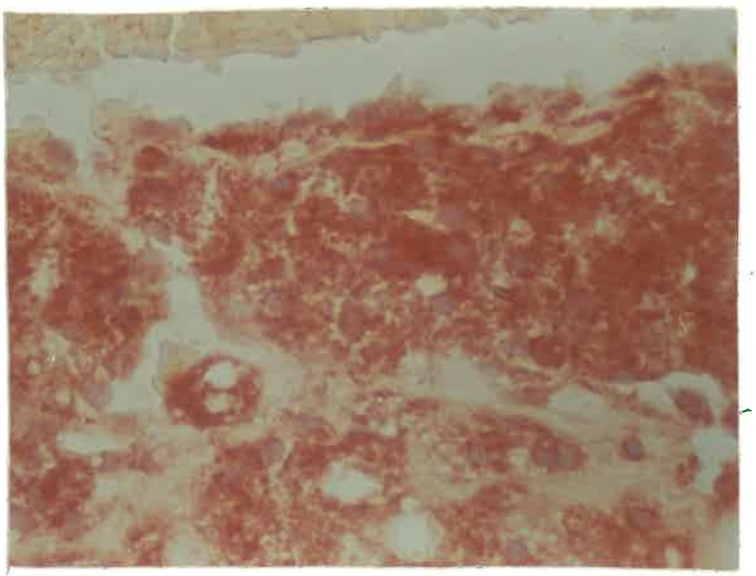


Fig. 34A. The edge of an organising plasma thrombus in a hypercholesterolaemic rabbit. Lipid containing macrophages are abundant. Scharlach R. x 510.

indicating that thrombolysis or fibrinolysis had been impaired; this was the third significant difference between the findings in normal and cholesterol-fed rabbits. Fourteen of the blocks contained lesions in which there were significant quantities of lipid in the form of foam cells; 4 lesions consisted almost exclusively of foam cells, but were distinguished from cholesterol-induced lesions by their larger size and the presence of new vascular channels - the latter were never seen in the foam cell lesions produced by cholesterol feeding alone. One final comment on the changes seen at 2 weeks. It seemed that the amount of metachromatic material in the pulmonary arteries, with and without thrombo-emboli was greater in cholesterol-fed rabbits than in animals on a normal diet.

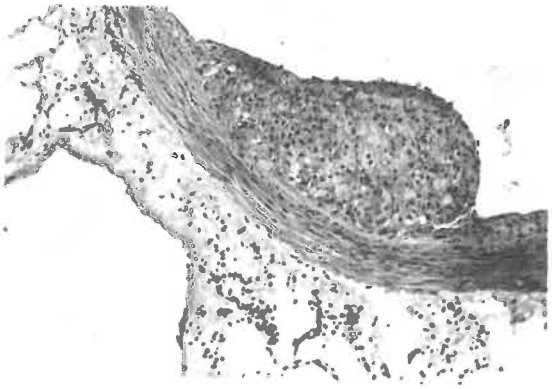
Four rabbits comprised the group examined 3 weeks after embolization. While the rate of thrombolysis was clearly impaired at 2 weeks after operation, by 3 weeks residual thrombus was no longer visible, suggesting that hypercholesterolaemia had only delayed this process by approximately one week.

At 3 weeks, lesions were seen in 19 blocks and in every instance significant accumulations of foam cells were present; 5 of the lesions consisted almost entirely of foam cells, and occasionally lipid containing cysts and clefts were seen. Many of the lesions at this stage were polypoid in appearance, comparable to the findings in the rabbits on a normal diet. A surprising finding was the absence of calcification in all lesions examined at 3 weeks. The differences at this stage when compared with the organization

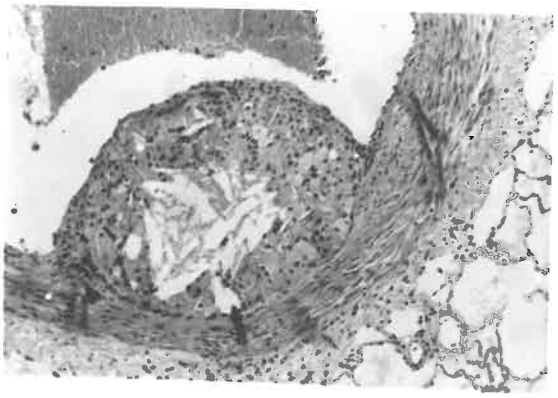
in normal rabbits were twofold; firstly, the greater lipid content in the hypercholesterolaemic rabbits; and secondly, the larger size of the resulting lesions.

Four weeks after embolization, the lesions were smaller. The average number of blocks of tissue showing residual lesions was 4.3, a finding comparable with the corresponding stage in normocholesterolaemic rabbits. Seventeen lesions were found, and they were confined to 3 animals, the fourth (8/CH) showing no demonstrable residual pulmonary artery lesions. It is of interest that the terminal total serum cholesterol level (580 mg./100 ml.) in this animal was much lower than in the other 3 rabbits (Table 5). Significant accumulations of foam cells were present in all the lesions, and cholesterol clefts were seen in some. Considerable calcification was noted; in those plaques with calcification the foam cell mass was relatively smaller. Additionally, the calcified lesions appeared larger than those without calcification. In one instance one of the larger foam cell plaques had ruptured through the media into the adventitia.

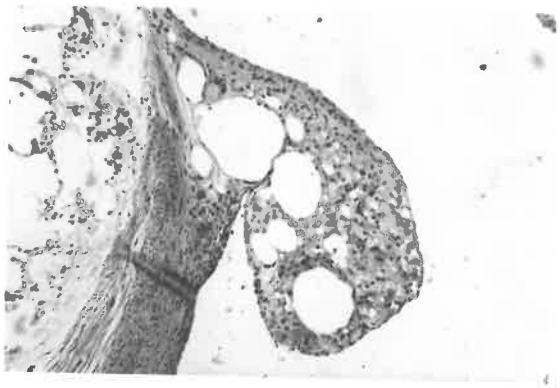
The final group of rabbits with plasma thrombo-emboli were examined 8 weeks after operation. Many of the lesions were still polypoid (Fig. 34D) and medial metachromasia was still prominent. At this stage the modifying effects of a cholesterol-rich diet appeared threefold. Firstly, the frequency of calcification in the resulting plaques was greater than in normocholesterolaemic rabbits. Secondly, there was clearly a greater quantity of lipid in the lesions



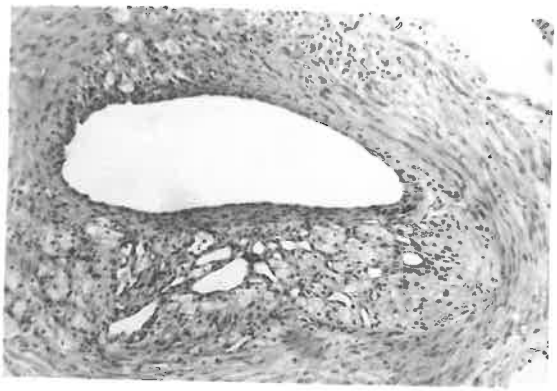
B.



C.



D.



E.

Fig. 34. Four lesions at 8 weeks after embolisation of plasma thrombi in hypercholesterolaemic rabbits. H. & E. x 90.

(Fig. 34E). Five lesions consisted almost entirely of lipid with only a thin superficial cap of fibrous tissue (Fig. 34B) and again acicular cholesterol clefts were visible (Fig. 34C). All 20 lesions at this stage contained a significant amount of lipid, while in only 4 of the 16 lesions in the corresponding normocholesterolaemic group was lipid present. It is perhaps significant that the experiments in which the most prominent lipid lesions were present were found in those animals with the highest serum chdesterol levels. In one instance, rupture of a plaque had occurred with extrusion of foam cells through the thinned media into the adventitia. Thirdly, the resultant lesions were larger in the cholesterol-fed rabbits than in those at a corresponding stage on a normal diet.

Fate of whole-blood thrombo-emboli

At 1 week after embolization the rate of endothelialization in this group was comparable to that in the other experimental groups. Even at this stage, foam cells were more prominent than in the organizing whole-blood thrombo-emboli of normocholesterolaemic rabbits. An obvious and striking feature in this group was the large number of lesions showing calcification which in many instances was extensive, some of the thrombi being almost completely replaced by calcified material. Despite the comparable total serum cholesterol levels, the frequency and extent of calcification was also clearly greater than in the hypercholesterolaemic rabbits with plasma thrombo-emboli.

Two weeks after operation, 4 of the 17 thrombi seen contained considerable quantities of residual thrombus, a finding which is consistent with the trend shown in the hypercholesterolaemic plasma-thrombus group, and supports the suggestion that cholesterol feeding, with a resultant hypercholesterolaemia and hyperlipaemia retards the process of thrombolysis. Calcification was again frequent, being present in many of the thrombi. Foam cells were prominent and in most of the thrombi were the major constituent. A little fibrous tissue was present at the periphery of the lesions, but at this stage the resulting lesions consisted essentially either of lipid, calcified material, or an admixture of both.

Only 9 lesions were detected at the stage 3 weeks after embolization, and these all consisted predominantly of foam cells. At this stage no calcified lesions were seen, but medial calcification was observed in many of the arteries in one experiment. This was the only occasion in the entire study, including rabbits on a normal diet, when this finding was noted.

In the group 4 weeks postoperatively, 12 lesions, confined to 3 of the 4 animals examined were noted. All 12 lesions contained significant quantities of lipid, and the two most striking lesions were seen in experiment 54/CH (Figs. 35 and 36). The birefringent nature of the lipid in one of these lesions shown in Figures 35A and 35B is illustrated in Figure 35C. The quantity of lipid in these lesions was clearly greater than that seen in the normocholesterolaemic

Fig. 35A. Macroscopic view of a fibro-fatty lesion.

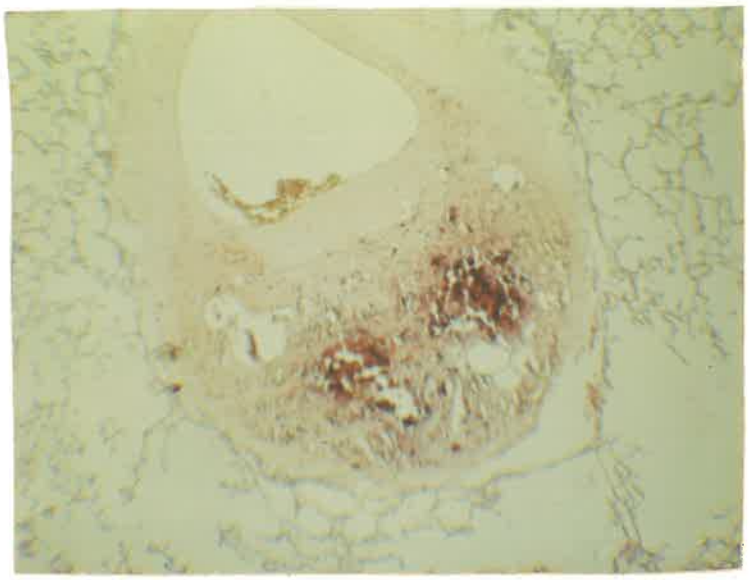
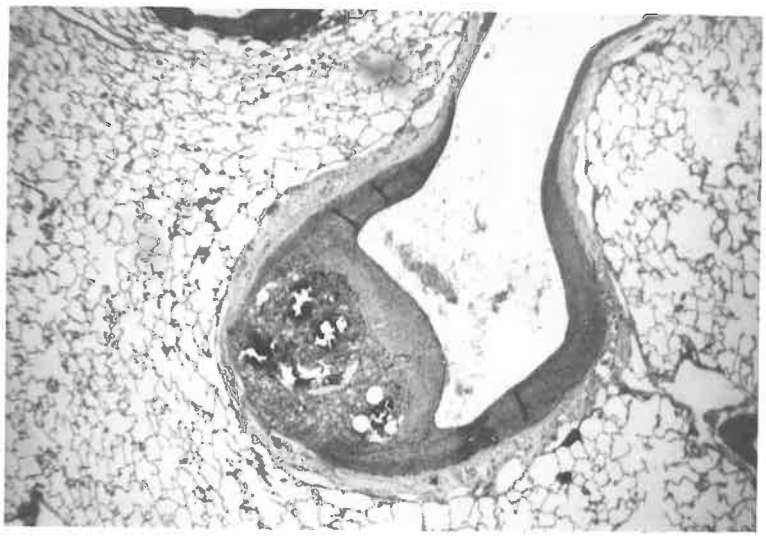
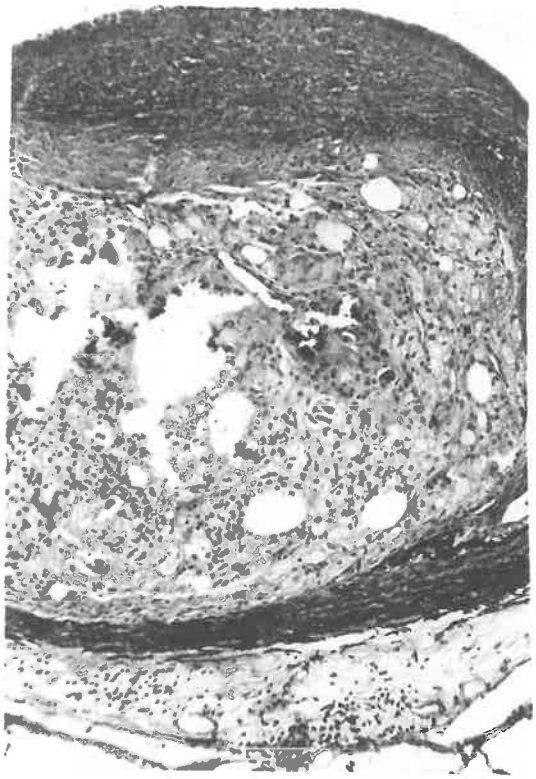


Fig. 35B. Same lesion as in Figure 35A. Scharlach R. x 30.

Fig. 35C. Same lesion as above. Note the birefringent nature of the lipid. Scharlach R. x 30.



A. H. & E. x 30.



B. Gomori elastic. x 120.

Fig. 36. A plaque at 4 weeks after embolisation.

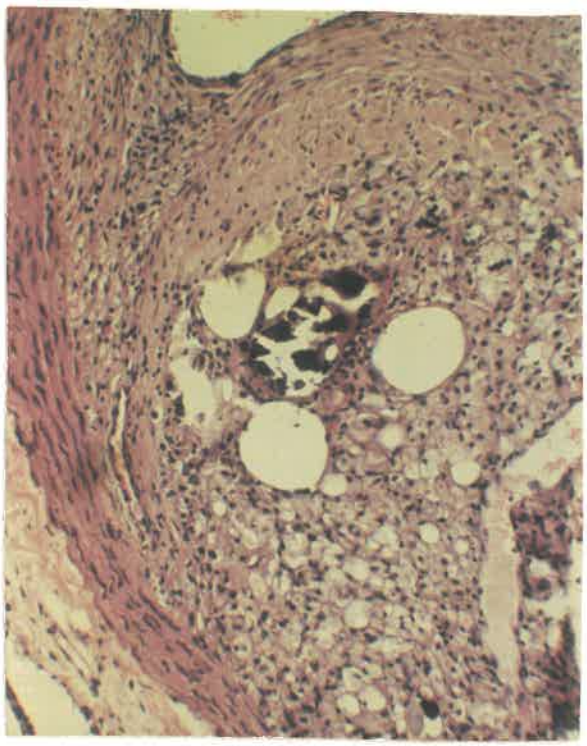


Fig. 36C.
H. & E. x 110.

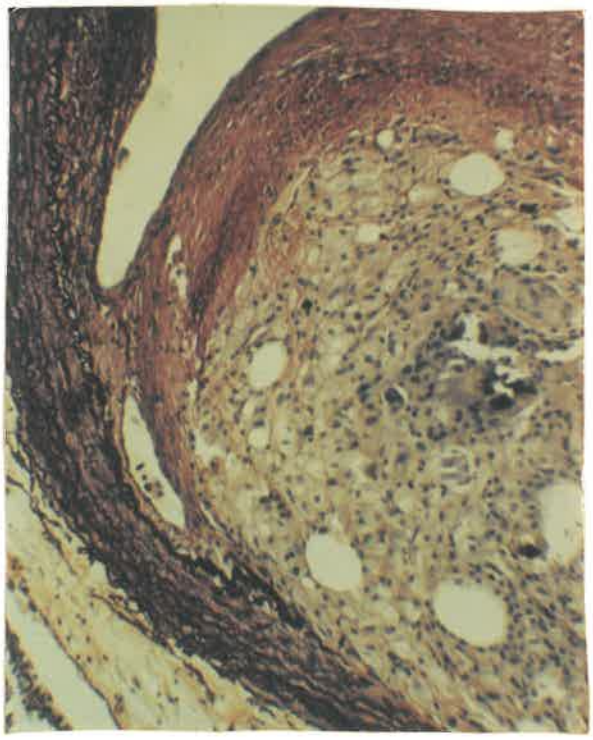


Fig. 36D.
Gomori
elastic
x 110.

animals, thus illustrating the additional contribution made by hypercholesterolaemia to plaque lipidization. Elastosis of the fibro-muscular cap can be seen in one of the lesions (Fig. 36B, D). Smooth muscle cells in the superficial aspect of this plaque have resulted in a complete ring of these cells surrounding the lumen of the vessel - this presumably represents an attempt to restore vascular function and morphology.

The final group of 4 rabbits was studied 8 weeks after embolization. Generally, the relentless and progressive nature of the process of organization was reflected in the smaller size of most of the lesions. Eight lesions were seen, and of these, 4 contained significant quantities of lipid (Figs. 37 and 38). The lipid content of these plaques was clearly greater than was observed in the comparable group of animals on a normal diet. Two lesions contained large quantities of calcified material (Fig. 39).

Cholesterol feeding has, therefore, clearly increased the lipid content of the plaques resulting from the organization of plasma and whole-blood thrombo-emboli. In most groups the frequency and extent of calcification was enchanced. In the process of organization, it was apparent that thrombolysis, probably due to impaired fibrinolysis was slower.

3. Discussion

In this study cholesterol-feeding was commenced 3 weeks before the introduction of the pulmonary thrombo-emboli, and

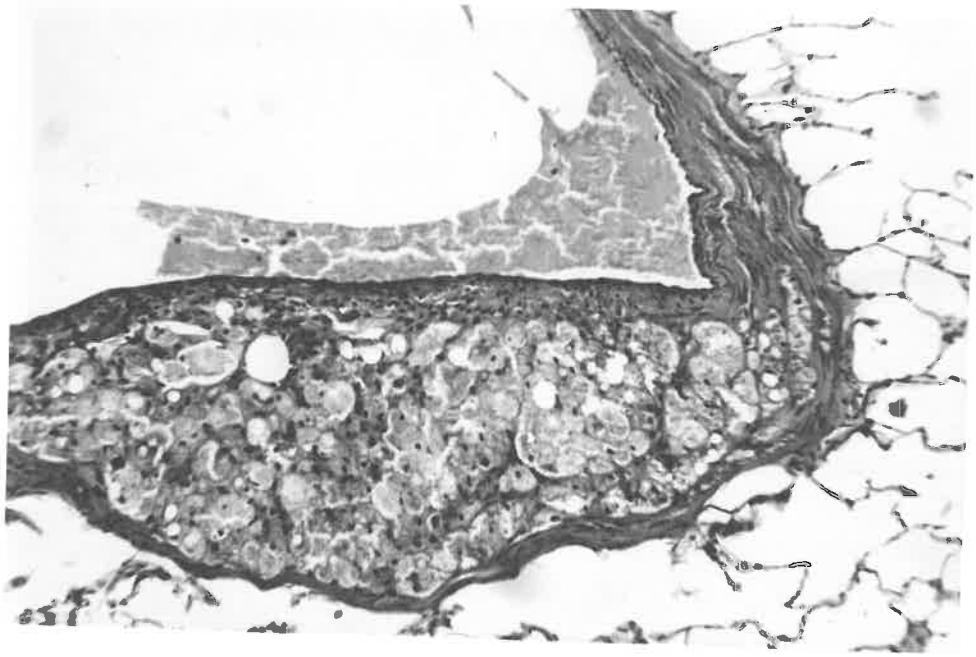


Fig. 37. At 8 weeks after embolisation of a whole blood thrombus in a hypercholesterolaemic rabbit. H. & E. x 150.

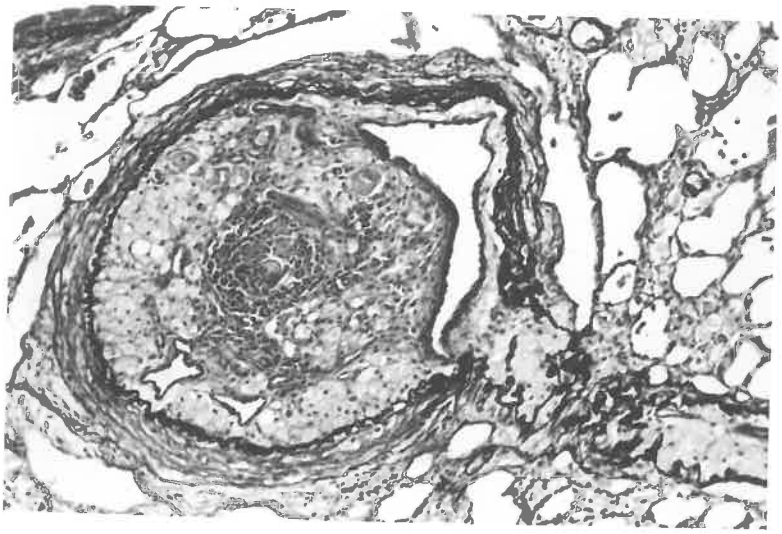


Fig. 38. 8 weeks. Gomori elastic. x 120.

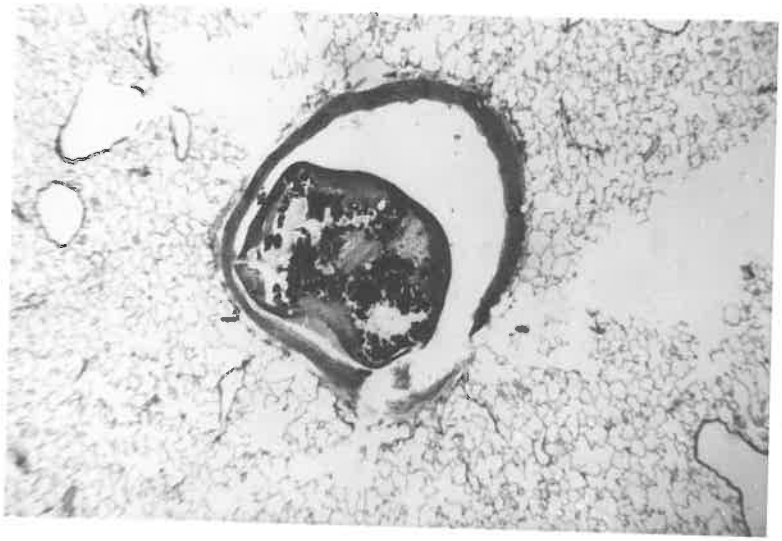


Fig. 39. 8 weeks. H. & E. x 30.

Two plaques in hypercholesterolaemic rabbits at 8 weeks after embolisation.

was continued for a maximum period of only 7 weeks. This limited cholesterol-feeding regime was used in a successful attempt to avoid the development of large or extensive pulmonary artery lesions which might have made the interpretation of the results of organization of thrombo-emboli difficult. The resulting lesions were typical of those induced by hypercholesterolaemia in the rabbit, and consisted predominantly of subendothelial foam cell accumulations with a variable but usually inconspicuous fibrous component.

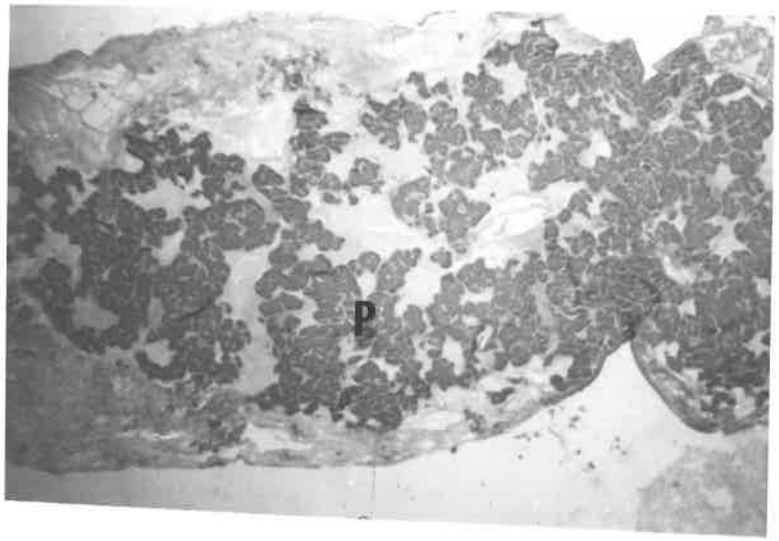
There have been conflicting views on the manner in which these foam cell lesions develop. Duff (1936), and Hueper (1944), believed cholesterol to be precipitated from tissue fluids in the vessel wall and then taken up by intimal macrophages. Altschul (1950) proposed that it was taken up by the lining endothelial cells and then carried by them into the intima. Leary (1941), on the other hand, considered that circulating foam cells, probably of hepatic origin, adhered to the endothelium which they then invaded. Macrophages have certainly been seen between endothelial cells (Poole and Florey, 1958) but whether they were entering or leaving the intima could not be determined. Rannie and Duguid (1953) supported Leary's view, but stated that instead of invading the vessel wall, the foam cells were covered by endothelium, thus becoming passively incorporated into the vessel wall. My observations support this latter view, but do not preclude the possibility of other contributing mechanisms. Occasional foam cells were seen deep within the media and their presence in this site cannot be explained by the above mechanism.

The focal nature of the lesions about the origins of branch vessels is interesting, and may well be determined by the pattern of blood flow at these sites.

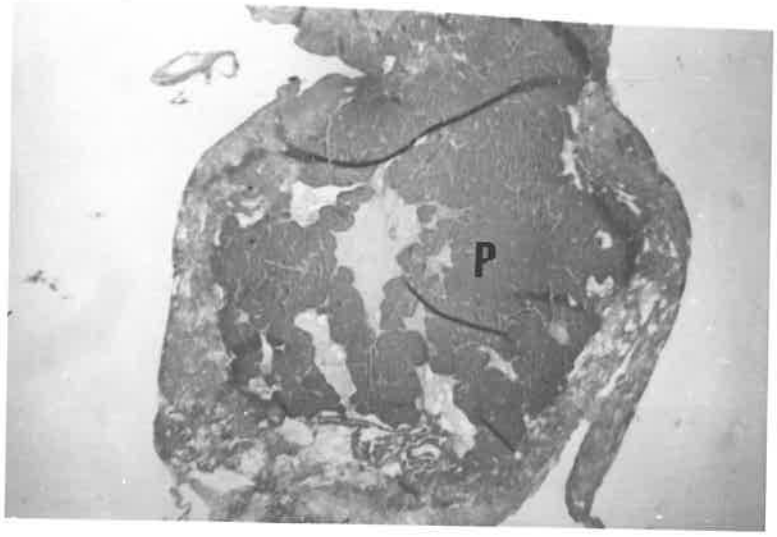
Many phases in the organization of both plasma and whole-blood thrombo-emboli were similar in hyper- and normocholesterolaemic rabbits at comparable times after embolization. No discernible difference in the rate or extent of the mononuclear or polymorphonuclear leucocytic infiltration was observed, while the process of endothelialization appeared comparable in both groups. Phagocytosis of cells including platelets and leucocytes, and vascularization were again in evidence. Hypercholesterolaemia did not appear to retard the process of fibrosis in the organizing thrombo-emboli, as was described in relation to the repair of intimal injury in rabbits by Prior and Hartmann (1956).

Enhanced lipid content

Early in the process of organization, it became apparent that the number of lipid-bearing macrophages was clearly greater in hypercholesterolaemic than in normocholesterolaemic rabbits. This difference was maintained throughout the duration of organization and was prominent in the resultant end-stage fibrofatty plaques. Several factors may be responsible. Firstly, it is possible that the thrombi derived from hypercholesterolaemic rabbits contained greater numbers of platelets than the equivalent thrombi derived from rabbits on a normal diet; this possibility was supported from a study of thrombi sectioned serially (Fig. 40).



A.



B.

Fig. 40. Chandler plasma thrombi from hypercholesterolaemic rabbits.
P = platelet aggregates. H. & E. x 30.

Two reports are relevant to this observed difference in platelet content. Firstly, Norday and Chandler (1964) demonstrated that a diet containing cholesterol and saturated fat increased the incidence of ADP-induced thrombosis in rats. Secondly, Silver and her colleagues (1964) have reported that cholesterol-fed rabbits develop a haemolytic anaemia - an increased release of ADP from the red cell may relate to the greater platelet content of thrombi in hypercholesterolaemic rabbits. Secondly, the lipid content within the platelet or in its "plasmatic atmosphere" may be elevated (Mustard et al., 1961). A third, and probably important mechanism contributing to the greater lipid content of the organizing thrombi could be the lipid-rich plasma trapped within the emboli. Using the Coon's indirect fluorescent antibody technique, Woolf and his colleagues (1966) concluded that lipid is present within the substance of thrombi in the form of lipoproteins and suggested that this lipoprotein may make a contribution to the lipid content of plaques resulting from their organization. Friedman and Byers (1965) have also claimed that the leakage of cholesterol rich plasma from newly formed vessels in the process of organizing a thrombus may contribute lipid. This claim could be tested by studying the organization of fibrin alone in hypercholesterolaemic rabbits. Finally, circulating foam cells in the cholesterol-fed rabbits were seen to adhere to the organizing thrombi. These may also have contributed slightly to the additional lipid content of the organizing

thrombo-emboli. While the platelet has undoubtedly played an important role in foam cell formation, it must be apparent that a number of other factors have also contributed, and it is possible that the amount of lipid derived from the phagocytosis of polymorphonuclear leucocytes has been underestimated.

Impaired thrombolysis

The rate of thrombolysis was significantly impaired in hypercholesterolaemic rabbits. Thrombolysis appears to be a composite of several mechanisms including proteolysis by leucocytes, fibrinolysis due to activation of plasminogen, phagocytosis of cells and phagocytosis of fibrin. Fibrinolysis, as evidenced by clear spaces in the thrombo-emboli was topographically related to invading endothelial cells, polymorphonuclear leucocytes, and possibly some undifferentiated mononuclear cells. The fibrinolytic activity of vascular endothelium has been well described by Warren (1964, 1965). Kwaan and McFadzean (1957), Scott and Thomas (1957), and Greig and Runde (1957), have all shown that various lipids can inhibit fibrinolysis, observations which are consistent with the impaired in vivo thrombolysis noted in this study.

Enhanced calcification

The overall frequency of blocks of lung tissue containing lesions showing calcification was some 2-3 times greater in cholesterol-fed animals than in those on a normal diet. This was true for both plasma and whole-blood thrombo-emboli. The reason for this greater calcium

content of the organizing thrombi in hypercholesterolaemic rabbits is obscure. These differences were observed at all stages of organization. The influence of cholesterol-feeding on calcium metabolism and on the tissue fixation of calcium clearly merits attention in view of these findings.

End stage lesions

In experiment 1 it was shown that the organization of autologous artificial pulmonary thrombo-emboli resulted in many instances in the formation of complex fibrofatty plaques with many of the histological features of human atheroma. The important role of platelet and probably polymorphonuclear leucocyte phagocytosis as a source of lipid in the plaques was stressed. Where platelets were sparse or absent, and fibrin the principal component of the thrombo-emboli, fibrous intimal thickenings with little or no lipid were the result. The end stage lesions in this study in hypercholesterolaemic rabbits differed in their greater lipid content, and a more frequent and extensive calcification. In conclusion, the effect of dietary induced hypercholesterolaemia on the resulting lesions has been to enhance significantly the lipid and calcium content of plaques.

Experiment 3: Electron microscopical observations on the organization of artificial plasma thrombo-emboli in the pulmonary artery of the rabbit

The purpose of this study was to define more precisely at the cellular level, the series of events by which the experimental thrombi produced in the Chandler apparatus are organized. The experiment was conducted in association with Dr. J. R. Casley-Smith from the

TABLE 8

MACROSCOPIC FINDINGS IN THE LUNGS OF THE 7 ANIMALS EXAMINED WITH THE ELECTRON MICROSCOPE. THE TIME AFTER OPERATION AT WHICH EACH ANIMAL WAS EXAMINED IS INDICATED.

Experiment number	Time (weeks) after operation	Macroscopic findings
50	4	One small lesion and some thickened vessels
51	4	No lesions seen
52	4	No lesions seen
53	2	One embolus found
54	2	Two emboli found
55	1	Small pulmonary infarct plus emboli
56	1	Large pulmonary infarct plus emboli

Electron Microscope Unit, University of Adelaide, with the technical assistance of Miss V. D. Soady and Mr. B. R. Dixon.

1. Materials and Methods

Seven male rabbits of mixed breed, fed ad lib. on a normal pellet diet were used. The operative procedure and the method of production of the artificial thrombi were as described in experiment 1. The rabbits were killed at intervals of 1, 2 and 4 weeks after the introduction of the pulmonary thrombo-emboli as shown in Table 8. The lungs were rapidly removed en masse, and inflated with freshly prepared 4% formaldehyde in Caulfield's (1957) medium. After fixing for half an hour at 2°C., they were cut into 2 mm. thick slices from the periphery to the hilum. Areas containing emboli were removed, and the tissue divided into blocks of approximately one cubic millimetre. These were post-fixed in 2% Osmium tetroxide in Caulfield's medium for one hour at 2°C. They were then dehydrated and embedded in araldite by the usual techniques. Sections were stained with lead (Reynolds, 1963) and examined in a Siemens Elmiskop I on carbon-collodion films or unsupported. To facilitate the difficult orientation in blocks of this type, numerous thick sections were examined by phase contrast.

2. Results

One week

With the light microscope the picture of the organizing plasma thrombi one week after embolization was the composite of a number

of processes, some of which were in evidence as early as 1½ hours after operation. Phagocytosis and fibrinolysis had reduced the size of many of the emboli. Endothelialization was complete or nearing completion. Other features included platelet phagocytosis and variable foam cell formation, a mixed mononuclear infiltration of the thrombus, fibroblastic invasion with fibrosis, some vascularization, slight elastic fibre formation, and copious debris from degenerate polymorphonuclear leucocytes. These facets have all been illustrated in Experiment 1.

Platelets were not conspicuous with the electron microscope at this stage, and could only be recognized with difficulty, most having degranulated (Fig. 41). Platelets in the thrombi even before embolisation showed considerable degranulation. In the thrombo-emboli they were enmeshed in a material, some of which exhibited the characteristic cross-banding of fibrin, but most of which was amorphous. It occurred in clumps and bands with intervening clear spaces (Fig. 41). Thrombolysis (fibrinolysis) was evident surrounding some endothelial cells (Fig. 42) and macrophages; a few of the latter had ingested fibrin (Fig. 43).

Both monocytes and macrophages were present in large numbers. (The term "monocyte" has been reserved for those cells similar to circulating monocytes, and the term "macrophage" is used to designate those cells with more cytoplasm, inclusions, and pseudopods. There is good evidence, however, that one form may

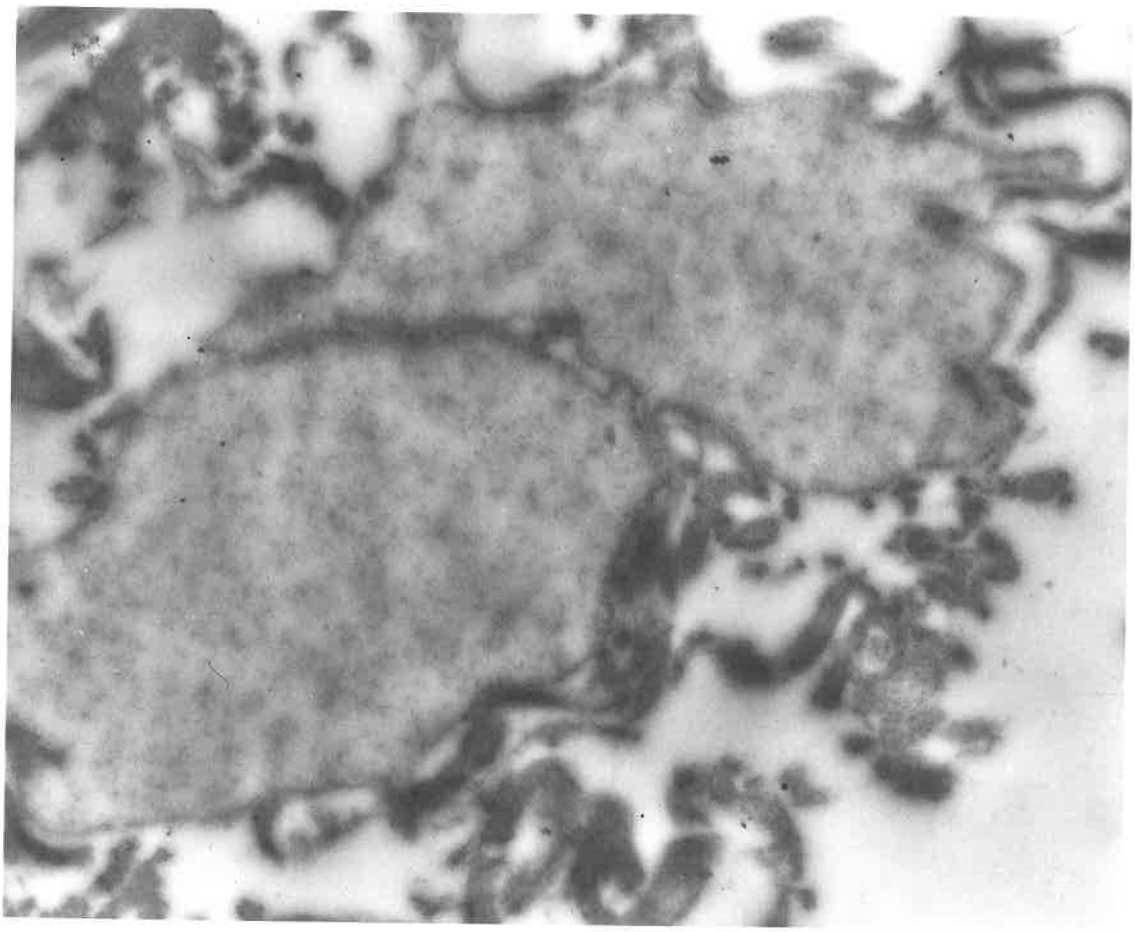


Fig. 41. One week after embolisation. Two adjacent platelets showing advanced degranulation, enmeshed in fibrin. (x 24, 000)

Fig. 42. One week. The thrombus (T) can be seen to consist of many amorphous areas, interspersed with pale regions. A further pale region is present bordering the endothelium (E); this probably represents endothelial fibrinolysis. The endothelium can be seen to contain many ribosomes and some moderately dilated endoplasmic reticula. (x17,000)

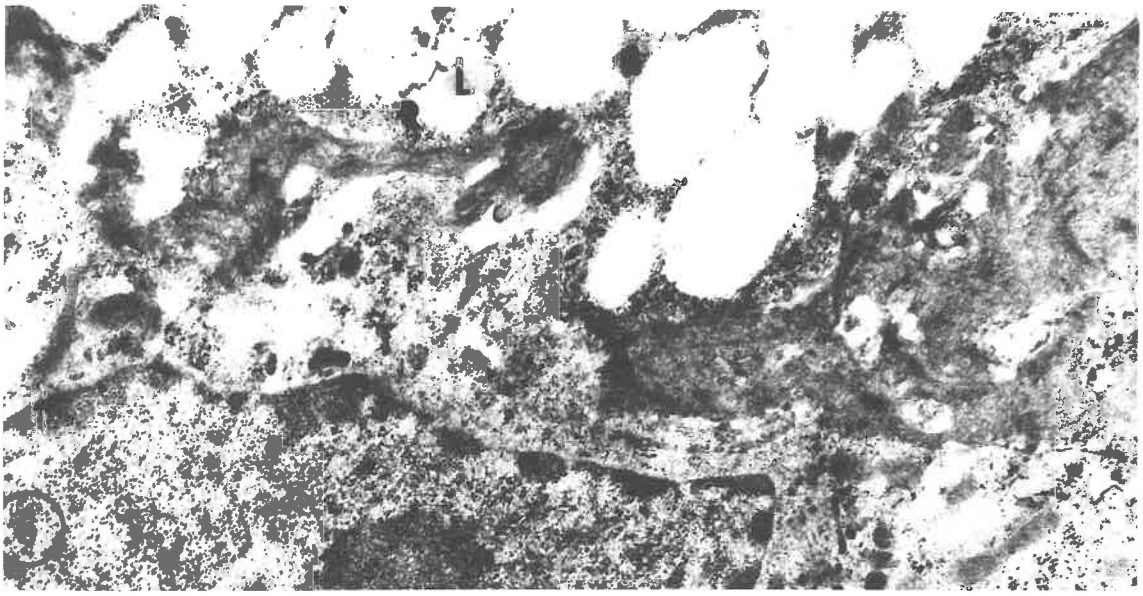


Fig. 43. One week. A portion of a macrophage is visible. Fibrin can be seen within the cell after ingestion (F). Some pale vesicles, which presumably contain lipid (L), may also be seen. (x25,000)

transfer to the other.) Few granulocytes were left at one week. Other cells, very similar to endothelial cells lay adjacent to the endothelium. These may have arisen from migrating endothelial cells. Because of this endothelial migration, it is possible that some of the other cells within the thrombus, designated as "monocytes" and "fibroblasts" may have had an endothelial origin.

On its free surface the thrombus was lined by flattened endothelial-like cells. With the light microscope these appeared to arise from a growth of the adjoining vascular endothelium, but the probability that circulating mononuclear cells are also implicated in the process of endothelialization was suggested (experiment 1). The electron-microscopic appearances have not precluded either origin.

The cells of the organizing thrombus contained some inclusions in large cytoplasmic vesicles. This was particularly true of the macrophage-like cells (Fig. 43), but they were also seen in the monocytes and endothelium. There was evidence of some ingestion of fibrin, platelets, erythrocytes, granulocytes and general cellular debris. Most of the inclusions were electron-transparent vacuoles measuring 0.1-0.5 μ., which almost certainly had contained lipid before removal by the embedding process (Casley-Smith, 1963, 1967). Another notable feature was the unusual thickness of the endothelial cells. Both they (Fig. 44) and the monocytes contained many ribosomes and dilated endoplasmic reticula, filled with a moderately electron-opaque material.

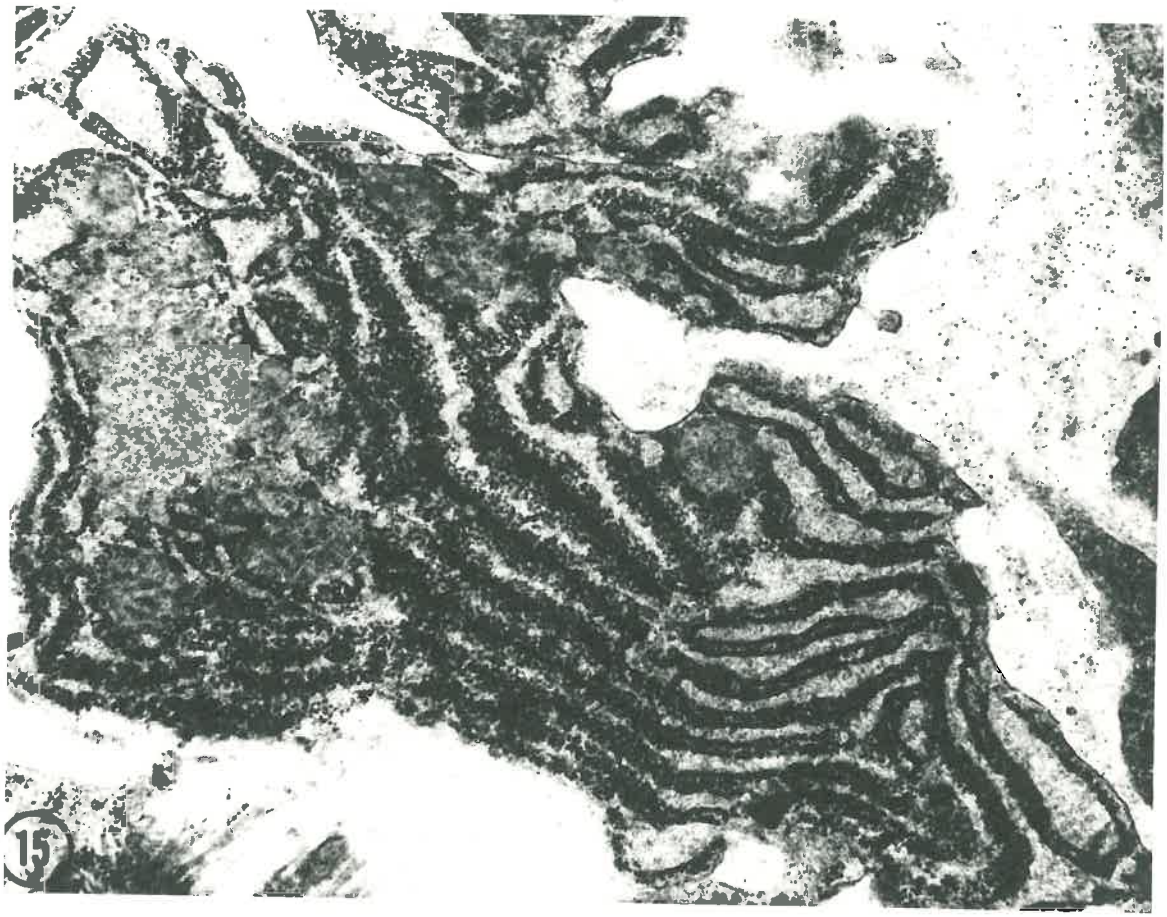


Fig. 44. One week. Endothelial-like cells containing dilated endoplasmic reticula and many ribisomes. (x28,000)

Two weeks

Progressive organization with fibrosis was apparent with the light microscope at this stage, and foam cells were visible (experiment 1).

With the electron microscope, the appearances had altered somewhat from the findings at one week. A few capillaries were seen within the organising thrombi, and the endothelial cells within the thrombi often contained inclusions similar to those within the monocytes and macrophages. These endothelial cells still had a dilated endoplasmic reticulum filled with grey material, and many ribosomes. The number of macrophages and monocytes had increased, and their inclusions were more numerous. The inclusions in the macrophages and also in some of the other cells were occasionally more complex, containing myelin forms and ferritin.

There was still much amorphous extracellular material present, but some collagen fibrils were now apparent. Platelets were no longer discernible, but cellular debris was abundant, especially towards the centre of the thrombi; some of this debris probably had a platelet origin.

Four weeks

With the light microscope the lesions at this stage showed copious fibrous tissue, but foam cells were also visible, sometimes copious and on other occasions sparse. Smooth muscle cells had appeared, particularly in relation to the new vascular channels

(experiment 1).

Considerable changes were also seen with the electron microscope. Many capillaries were present within the thrombi (Fig. 45). These had the typical "juicy" look of young capillaries (Cliff, 1963; Schoefl, 1963) with abundant cytoplasm, many ribosomes, and slightly swollen endoplasmic reticula. Some partly or completely open junctions were seen, and the basement membranes were poorly developed. The endothelial cells often contained inclusions (Fig. 45).

There were many bundles of collagen fibres, some of the earlier forms being much thinner than the older fibres (Fig. 45). Scattered between the collagen fibres were "fibroblasts" some of which were in close opposition to the endothelial cells, which they resembled. These fibroblasts, endothelial cells, the few monocytes, and the many macrophages all contained many lipid inclusions (Figs. 46 and 47). These were most numerous in the macrophages which had been converted to typical "foam cells", identifiable only by their borders of massed pseudopodia (Fig. 47). Some of these cells also contained variable electron-opaque deposits (Fig. 47) similar to, and probably consisting of lipofuscins. A few cholesterol clefts were seen (Fig. 46).

3. Discussion

In experiment 1 it was shown that the organisation of autologous plasma and whole-blood pulmonary thrombo-emboli in normo-

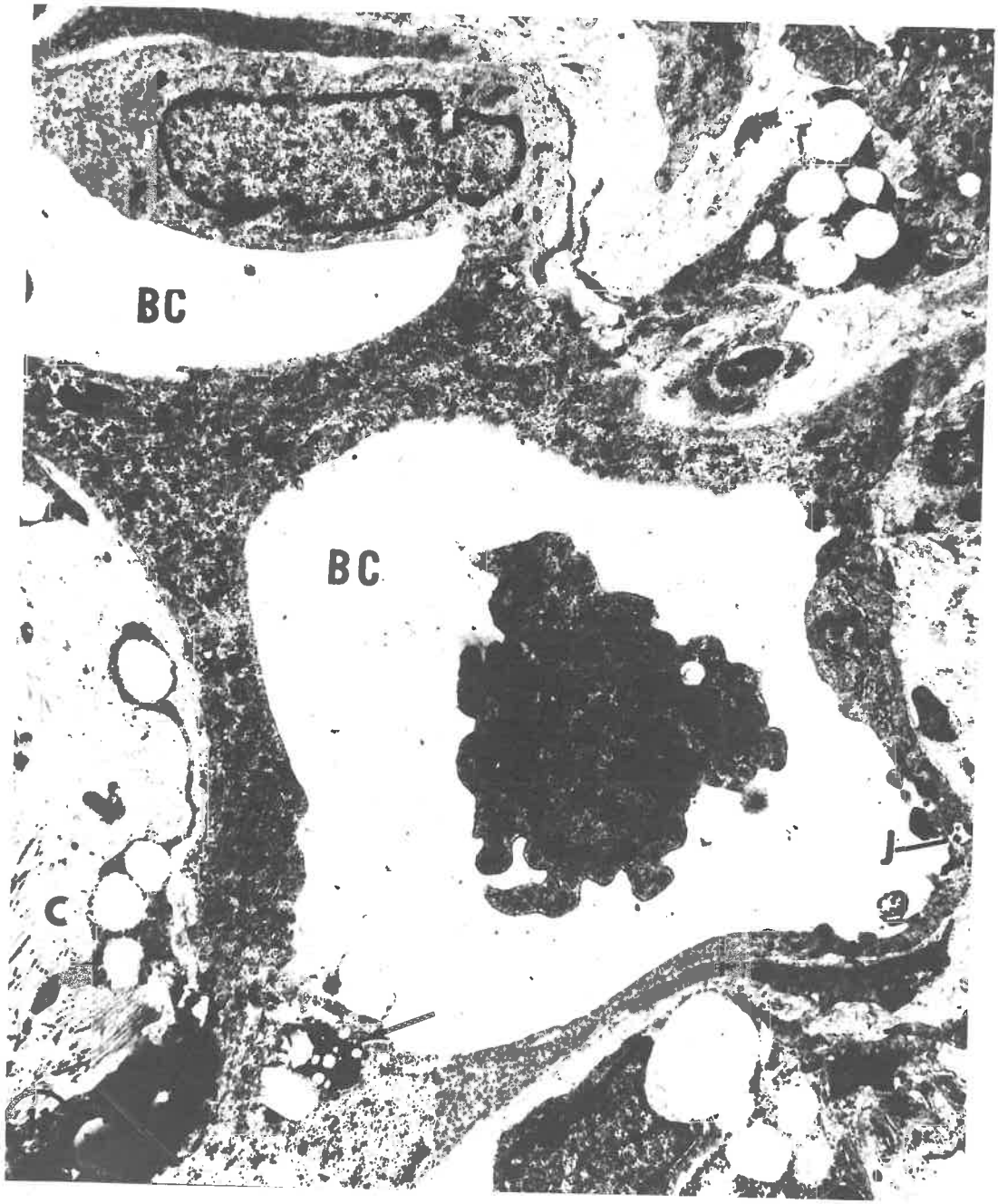


Fig. 45. Four weeks. Two blood capillaries (BC) are shown. There is some lipid and, probably, lipofuscin in the wall of one (arrow), which also shows a partly open junction (J). A number of cells are seen outside the capillaries; they are probably monocytes. One of the capillaries contains a monocyte. Collagen (C) is visible. (x10,000)

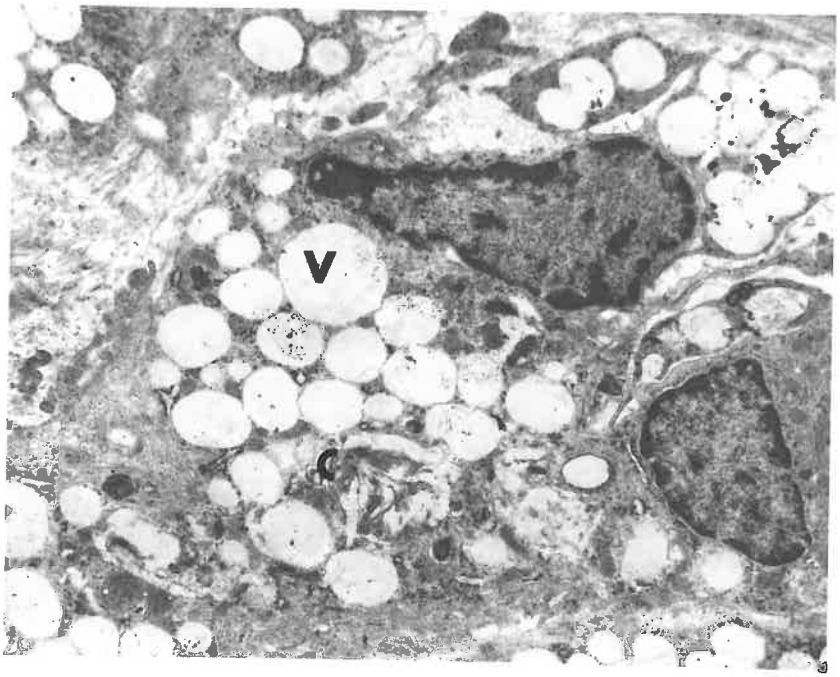


Fig. 46. Four weeks after embolisation. Macrophages containing abundant lipid vacuoles (V). A few cholesterol clefts (C) can be seen in one cell. (x 7, 300)

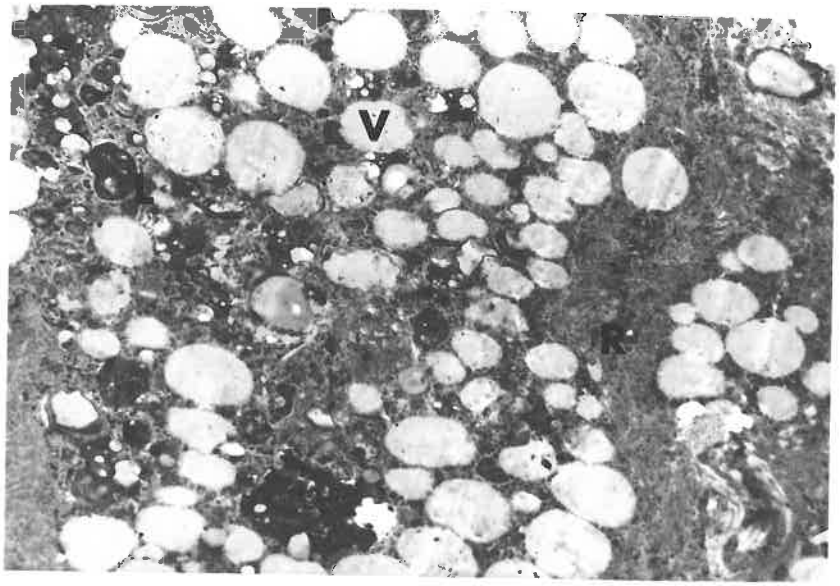


Fig. 47. Four weeks after embolisation. Macrophages containing abundant lipid vacuoles (V) and some electron-opaque deposits, probably lipofuscins (L). Numerous pseudopodia (R) at the margins of these macrophages can be seen. (x 4, 900)

cholesterolaemic rabbits ultimately resulted in the development of complex fibrofatty plaques with a remarkable histological similarity to the naturally-occurring atheromatous lesions of man. In accord with Hand and Chandler (1962), it was concluded that foam cells arose by the phagocytosis of platelets, and that the latter were an important source of lipid in these lesions. In this ultrastructural study, some evidence of platelet phagocytosis was seen, thus confirming the light microscopic observations. While platelets may well provide an important source of lipid in these organizing thrombo-emboli, other mechanisms, including the phagocytosis of degenerate polymorphonuclear leucocytes and red cell membranes, and synthesis by macrophages might also contribute. If polymorphonuclear leucocytes contain appreciable quantities of lipid which would not be surprising in view of their activity in lipid synthesis, phagocytosis of these cells could make a significant contribution to the total lipid of these lesions. This possibility should not, therefore, be overlooked.

While the possibility that the phagocytosis of fibrin might contribute to the thrombolytic process was suggested in experiment 1, this could not be demonstrated with the light microscope. In this study, however, a few macrophages containing fibrin were present one week after embolization. Thus it would appear that fibrin phagocytosis can complement the role of extracellular fibrinolysis in <u>in vivo</u> thrombolysis, although this mechanism is probably relatively unimportant. However, if the intracellular

breakdown of fibrin is rapid, one would not see much intracellular fibrin even in the presence of considerable fibrin phagocytosis, and perhaps intracellular distruction of fibrin is more important than the results suggest.

Lipid inclusions in large cytoplasmic vesicles were prominent in macrophages, and were also seen in monocytes and endothelial cells. Of particular interest was the presence of similar inclusions within the endothelial cells lining the new capillaries of the organizing thrombi.

These lipid inclusions became more numerous with the passage of time after embolization, and also became more complex with the emergence of some myelin forms, and ferritin. Additionally, some macrophages contained electron-opaque inclusions, probably consisting of lipofuscins and at 4 weeks a few cholesterol clefts were seen.

The light microscope observations in experiment 1 suggested a dual origin for the endothelium lining and invading the organizing thrombo-emboli. While the contribution made by an overgrowth of cells from the adjoining pre-existent vascular endothelium appeared to be an important, if not major, mechanism of endothelialization, it was suggested that the endothelial cells might also arise by the transformation of circulating mononuclear cells. This latter mechanism has been implicated in other situations, including the endothelialization of mural thrombi on artificial aortic grafts (Ghani and Tibbs, 1962). These conclusions receive further support

from the experimental observations of Halpert and colleagues (1966). In the present study the electron microscopic appearances have not been able to resolve this problem.

The transformation of endothelial cells into fibroblasts and macrophages, a possibility mentioned by Altschul (1950) and suggested by the light microscopic observations in experiment 1, received some support in this ultrastructural study in that a continuous series of cells could be seen from those typical of endothelial cells, to others with the features of fibroblastic and mononuclear cells. It should be emphasized, however, that the evidence is by no means conclusive. The marked thickness of the endothelial cells, which contained many ribosomes and a dilated endoplasmic reticulum filled with a moderately electron-opaque material is consistent with a considerable degree of metabolic activity in these cells, and this might be related inter alia to their fibrinolytic or phagocytic properties, and also perhaps to the formation of basement membrane, ground substance and collagen.

The demonstration of "juicy" young capillaries within the thrombi, in which some partly or completely open junctions were seen, and in which the basement membranes were poorly developed, indicates that the leakage of cholesterol rich plasma from newly formed vessels contributing lipid to plaques as claimed by Friedman and Byers (1965) may be possible.

In conclusion, this electron microscopic study has confirmed and extended some of the observations made with the light microscope

but has failed to resolve all of the questions raised by these earlier experiments. The involvement of monocytes and the phagocytosis of platelets in the genesis of foam cells has been confirmed, and the endothelial cell can probably transform into several other cells. The origin of smooth muscle cells in plaques remains undetermined. In a similar ultrastructural study on whole blood. Chandler thrombi, which has just come to hand (Still, 1966) it was concluded that lipophages were derived from monocytes and that phagocytosis of platelets was an important source of lipid.

Summary of Part II

The organization and ultimate fate of autologous plasma and whole blood pulmonary thrombo-emboli in rabbits on a normal diet has been studied at intervals ranging from 1½ hours to 18 weeks. Changes occurring during the organization of anterior eye chamber implants of plasma thrombi have also been described, and compared with the changes seen at comparable times in plasma thrombo-emboli.

The first response observed was an invasion by polymorphonuclear leucocytes, the extent of which was apparently related to the platelet content of the thrombo-emboli. As an explanation it was suggested that platelets might release a leucotactic factor. The author is of the opinion that this property of polymorphonuclear leucocyte attraction to platelets is of fundamental importance in the resolution of thrombi and haemostatic plugs and accounts for the characteristic disposition of leucocytes around the margins of platelet clumps.

Endothelialization of the surface of the emboli was evident by 3 days, and at 1 week was nearing completion. Two processes were involved; firstly, a growth of endothelium from the adjacent vascular endothelium; secondly, an endothelial transformation of mononuclear cells, possibly derived from circulating monocytes. The contribution of the latter to the process of endothelialization appeared to be relatively less important than the former. Consistent with this conclusion is the observation by Poole, Sanders and Florey (1958) that, even after 7 months, endothelium had still not completely covered an experimentally induced defect in the abdominal aortas of rabbits.

By 48 hours the thrombo-emboli had assumed a spongy appearance peripherally, with clear spaces near and around the polymorphonuclear leucocytes. Similar clear zones were later evident in relation to invading "endothelial" cells and were considered to reflect the fibrinolytic activity of these cells. Some fibrin phagocytosis was observed with the electron microscope.

After the leucocytic infiltration, a mixed population of mononuclear cells entered the thrombi, including lymphocytes, monocytes, and a third intermediate cell type, possibly of monocytic or lymphocytic origin. These latter cells increased in number, and with the ingestion of degenerate leucocytes, platelets and red cells, were ultimately transformed into lipid-containing foamy macrophages. It was concluded that the phagocytosis of platelets and probably also

of polymorphonuclear leucocytes, were the major sources of foam cell lipid. Some of these aspects were confirmed in an ultrastructural study of the organization of plasma thrombi.

Many aspects of the organization of plasma and whole-blood thrombo-emboli were comparable. Two important differences emerged; firstly, the greater frequency and extent of the polymorphonuclear leucocyte response in the whole-blood thrombi; and secondly, foam cells were clearly more numerous in the organizing whole-blood thrombi. It was considered that both differences might reflect the greater initial platelet content of the whole-blood thrombo-emboli. When platelets were sparse or absent, and fibrin the major component, organization resulted in a fibrous intimal thickening only. Other changes noted during the evolution of the thrombo-emboli included an increased interstitial metachromasia of the adjacent arterial wall, fibrosis, elastic fibre formation, the development of smooth muscle cells, calcification, and medial atrophy.

Many of the end-stage lesions derived from both plasma and whole-blood thrombo-emboli were complex eccentric fibrofatty plaques with a dense collagenous cap overlying a central lipid core, usually with lipid cysts and sometimes with acicular cholesterol clefts. Additional features included vascularization, calcification, and medial thinning, even to the point of aneurysmal dilatation. It was concluded that the organization of platelet rich autologous artificial thrombo-emboli leads to the production of arterial plaques

remarkably similar to the naturally occurring atheromatous lesions of man. These findings are considered to provide strong support for the thrombogenic hypothesis.

The influence of cholesterol feeding with its resultant hypercholesterolaemia and hyperlipaemia on the organization and fate of autologous artificial plasma and whole-blood thrombo-emboli in the rabbit was also investigated. It was found that many phases in the organization of the thrombo-emboli were comparable with those seen in normocholesterolaemic rabbits. In particular, no difference in the rate or extent of the leucocytic or mononuclear infiltration was observed. Nor was there any difference in the process or rate of endothelialization of phagocytosis. The extent of the resulting fibrosis was also comparable.

Thrombolysis, however, was clearly impaired in those animals on a cholesterol-enriched diet. It was suggested that this might reflect the inhibitory effect in vivo of hypercholesterolaemia or hyperlipaemia on the fibrinolytic mechanism.

Calcification was some 2-3 times more frequent and also more extensive, in the organizing pulmonary thrombo-emboli of the hypercholesterolaemic rabbits than in those on a normal diet. The reason for this finding remains obscure.

At all stages the number of lipid-bearing macrophages was clearly greater in the organizing thrombo-emboli in hypercholesterolaemic than in normocholesterolaemic rabbits. This difference was evident

in the end-stage complex fibrofatty plaques. The lipid-rich plasma trapped within the thrombo-emboli was considered to be an important additional source of lipid.

This study has shown that the end-stage complex fibrofatty plaques resulting from the organization of autologous artificial plasma and whole-blood pulmonary thrombo-emboli in cholesterol-fed rabbits are in many respects similar to the lesions produced in rabbits on a normal diet. The greater frequency and extent of calcification, and the greater lipid content of the lesions in hypercholesterolaemic rabbits have been emphasized. The resemblance of many of these plaques to the naturally occurring atheromatous lesions of man was striking. It is possible that hypercholesterolaemia in man might similarly modify the organization and fate of thrombi.

At the cellular level the same series of events was observed in the organization of plasma thrombi in the anterior eye chamber of the rabbit. Because these thrombi were mostly deficient in platelets further experimentation is necessary, but on a speculative note, one wonders whether the development of atherosclerosis is necessarily dependent on any properties inherent in the vascular wall.

A technique has now been developed by the author for the introduction of artificial thrombi into the systemic circulation of the rabbit and the fate of whole-blood thrombi in this situation is currently being studied in normocholesterolaemic and hyper-cholesterolaemic animals.

PART III

STUDIES OF PLATELET AGGREGATION

PART III - STUDIES OF PLATELET AGGREGATION

Platelet aggregation is important in the formation of the haemostatic plug and in thrombogenesis. In the past five years there has been a remarkable and rapid growth of interest in the phenomenon of platelet aggregation dating from Hellem's (1960) discovery of a red cell extract which could produce marked platelet clumping and which was identified in the following year as ADP (Gaarder et al., 1961).

In 1941, Wright described a quantitative technique for studying platelet adhesiveness. She added anticoagulated blood to a rotating glass container, and at intervals the number of platelets remaining in suspension were counted, a decrease in count indicating the number sticking to the glass. This principle formed the basis of many other techniques for measuring platelet adhesiveness, including the glass bead column introduced by Hellem (1960). More recently and almost simultaneously, Born (1962) and O'Brien (1962b) introduced a spectrophotometric technique in which platelet-rich plasma is stirred mechanically or electromagnetically, and the aggregation of platelets is measured by a fall in optical density. Modifications of this latter technique have been widely used, and the results to be described in the following experiments have all been obtained employing this method.

Experiment 4: The influence of catecholamines on nucleotide-induced platelet aggregation

1. Materials and Methods

Platelet aggregation was measured in a 'Unicam SP 400' spectrophotometer with the light source set at a wavelength of 600 mμ. Antecubital venous blood was obtained from healthy adult volunteers using siliconized glass syringes and needles. Nine volumes of blood was added to one volume of 3.8% trisodium citrate and platelet-rich plasma (PRP) was prepared by centrifuging at 350 g. for 20 minutes. Three millilitre aliquots of the PRP were added to siliconized glass cuvettes having a light path of 10 mm. The PRP was stirred electromagnetically. All test substances were prepared in 0.9% sodium chloride at such concentrations that the addition of 0.1 ml. to the cuvette would result in an appropriate final concentration. Experiments were carried out at room temperature maintained between 22-25°C.

2. Results

Platelet clumping activity of adrenaline, nor-adrenaline and dopamine

Both adrenaline (Evans Medical Ltd.) and nor-adrenaline (L. Light & Co. Ltd.) were found to induce platelet aggregation in citrated platelet-rich plasma. Dopamine (Nicholas Pty.), however, produced no effect. The degree of aggregation produced by adrenaline was greater than that produced by equimolar concentrations of nor-adrenaline. Aggregation began immediately after the addition of adrenaline or nor-adrenaline, but the initial rate and the degree of aggregation for both of these substances

was invariably less than that produced by equimolar concentrations of ADP (Sigma). Moreover, aggregation was often incomplete, even with maximal doses of catecholamine.

The clumping activity produced by a given concentration was found to vary in different subjects (Figs. 48 and 49), and also in the one subject from day to day. In all experiments, however, a final adrenaline concentration of 2.5×10^{-8} M was found to be inactive. Occasional subjects showed slight platelet aggregation with a final concentration of 2.5×10^{-7} M, while concentrations within the range 2.5×10^{-6} to 10^{-4} M invariably produced aggregation. The optimal concentration was usually of the order of 2.5×10^{-5} M, although a greater effect was occasionally noted with a final concentration of 2.5×10^{-4} M.

Although adrenaline-induced aggregation showed slight reversal after 30-60 minutes, little or no disaggregation was observed at 10 minutes, contrasting with that usually seen with the lower concentrations of ADP. Moreover, the addition of a second equimolar amount of adrenaline produced little or no further platelet aggregation.

Examples of adrenaline-induced aggregation are shown in Figures 48 and 49. Figure 48 shows a typical response to adrenaline at final concentrations ranging from 2.5×10^{-5} to 2.5×10^{-7} M. In this experiment, the optimal adrenaline concentration was 2.5×10^{-5} M, and a ten-fold increase in concentration (not plotted)

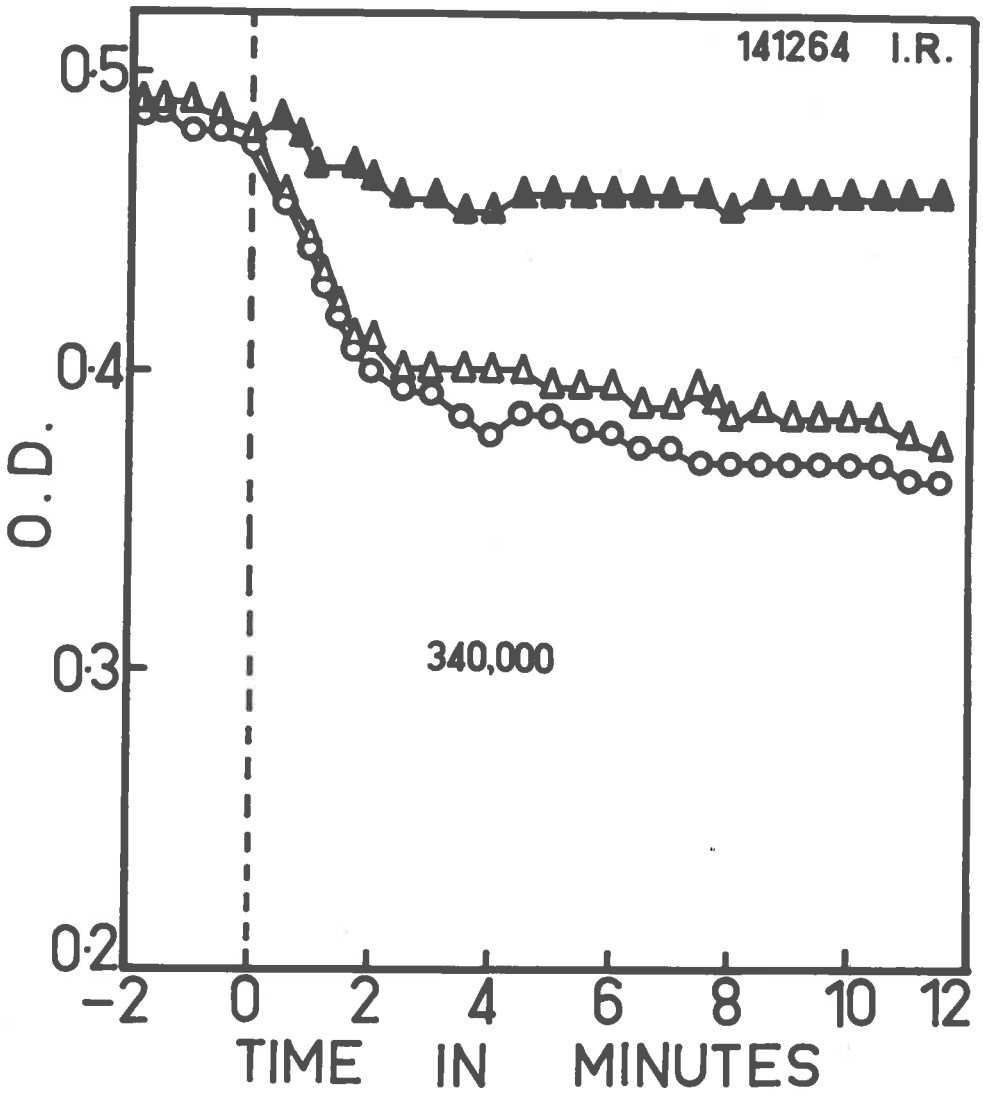


Fig. 48. Adrenaline-induced platelet aggregation. Adrenaline was added at 0 min. to give the following final concentrations: 2.5×10^{-7}M (▲), 2.5×10^{-6}M (△), and 2.5×10^{-5}M (○). In this subject the response is small. The optimal effective concentration was 2.5×10^{-5}M. A concentration of 2.5×10^{-4}M (not graphed) produced no greater effect.

In Figures 48-65 inclusive, O.D. indicates optical density of the plasma, and the platelet count is shown, in this instance 340,000 per c.mm.

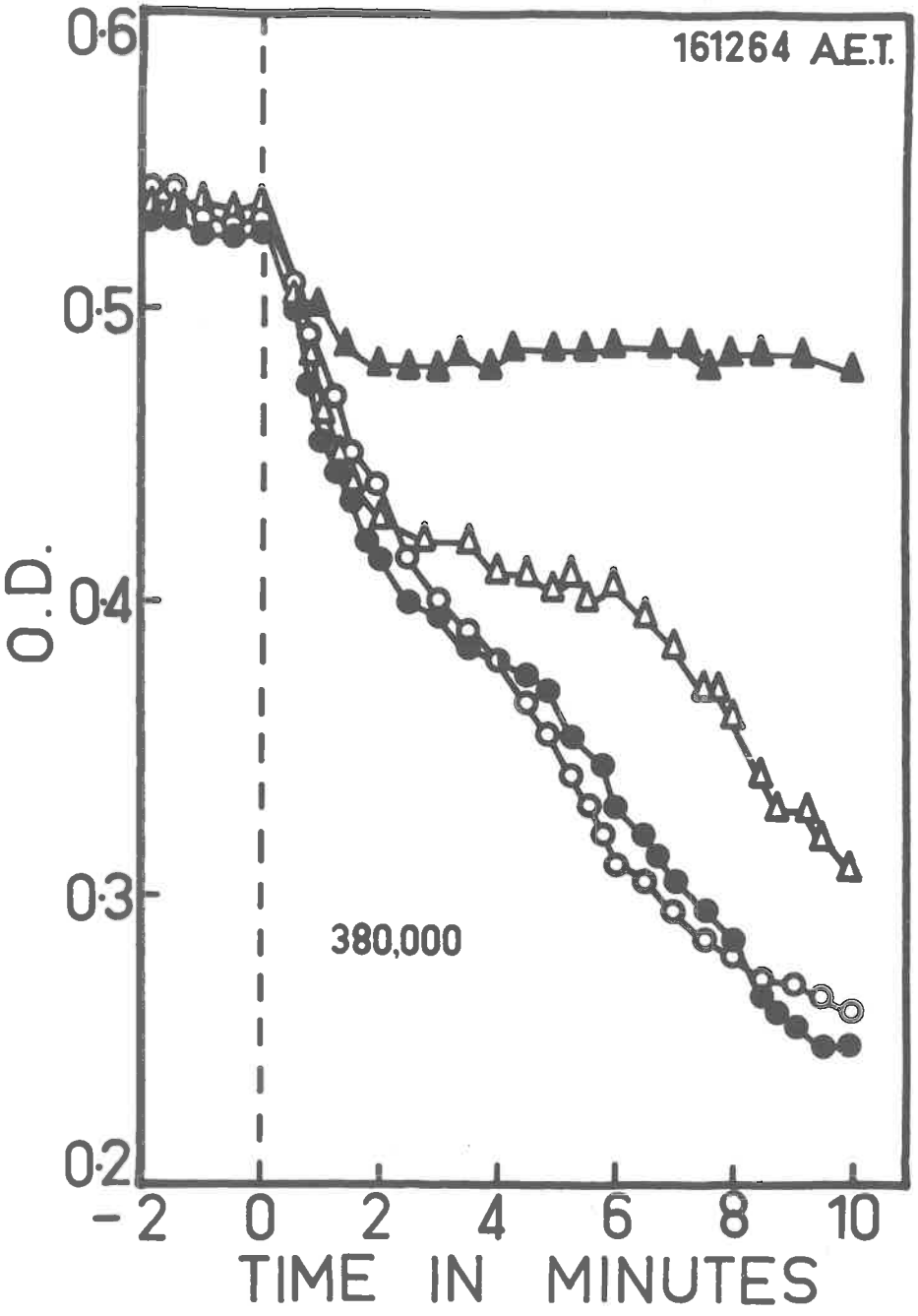


Fig. 49. Adrenaline-induced platelet aggregation. Adrenaline was added at 0 min. to give the following final concentrations: 2.5×10^{-7}M (▲), 2.5×10^{-6}M (△), 2.5×10^{-5}M (○), and 2.5×10^{-4}M (●). In this subject the response is marked. The optimal effective concentration is 2.5×10^{-5}M. Note the biphasic response.

resulted in no further aggregation. A similar optimal concentration can be seen in Figure 49. This figure also shows the much greater degree of aggregation noted in some subjects. A tendency to a biphasic response can be seen, a pattern observed only infrequently. In this subject (Fig. 49) it was most prominent with a final adrenaline concentration of 2.5×10^{-6} M.

The effect of adrenergic blocking agents

Phentolamine is considered to antagonise the action of catecholamines on α-receptor sites. At a final concentration of 2.5×10^{-4} M this substance completely inhibited the platelet clumping activity of equimolar adrenaline, the latter being added to the platelet-rich plasma 30 minutes after the phentolamine (Figure 50 - bottom). Further, the addition of phentolamine to plasma 30 minutes before the addition of ATP or ADP, both at a final concentration of 2.5×10^{-5} M, also resulted in an inhibition of platelet clumping, which was virtually complete for ATP (Figure 50 - top), but less complete for ADP. Studies with phenoxybenzamine, another α-receptor site antagonist, have so far been inconclusive.

Catecholamine potentiation of nucleotide-induced aggregation

Adrenaline, nor-adrenaline, and dopamine enhance the platelet clumping activity of ADP or ATP (Sigma) when either of the latter is added to citrated platelet-rich plasma 5, or 10.5 minutes after the catecholamine. In Figure 51, the effect of

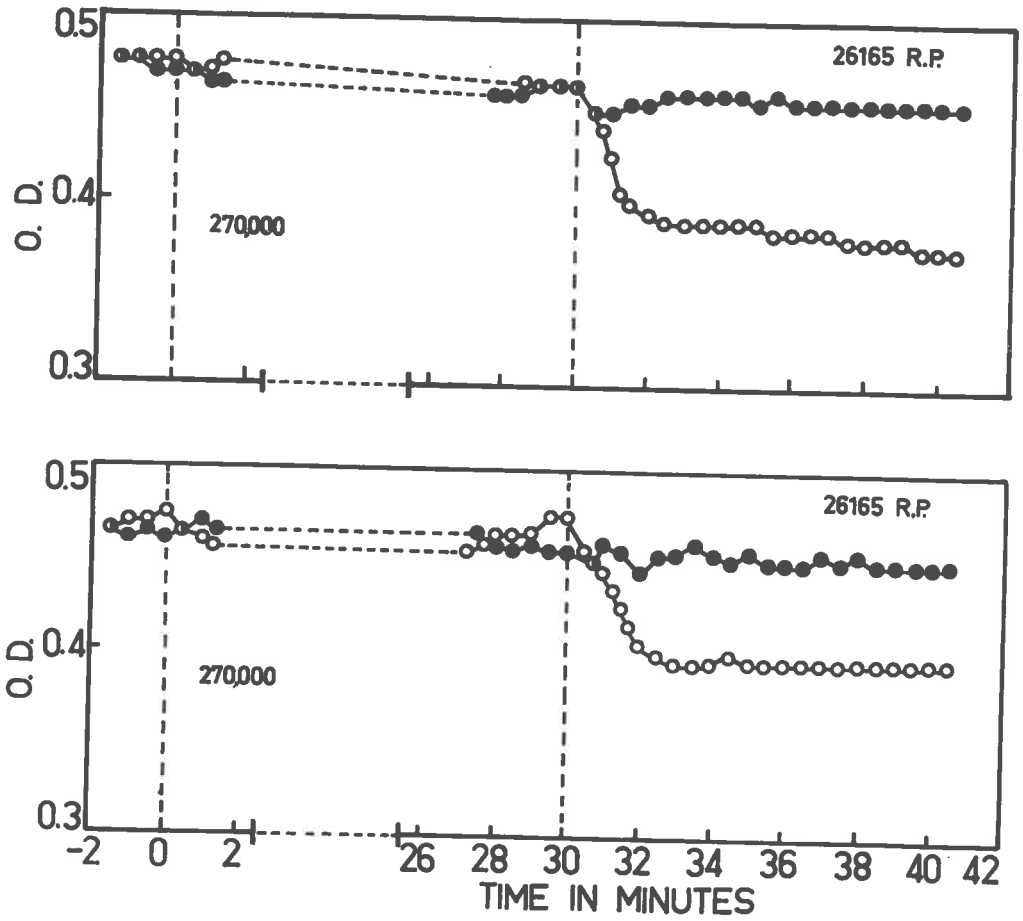


Fig. 50. The top graph shows phentolamine (2.5×10^{-4}M) added at 0 min., almost completely inhibiting the clumping activity of ATP (2.5×10^{-5}M) added at 30 min. (●). In the control experiment (⊙) saline was added at 0 min., and ATP (2.5×10^{-5}M) at 30 min.

The bottom graph shows phentolamine (2.5×10^{-4}M) added at 0 min., completely inhibiting the clumping activity of equimolar adrenaline added at 30 min. (●). Saline replaced the phentolamine in the control experiment (⊙) and adrenaline (2.5×10^{-4}M) was added at 30 min.

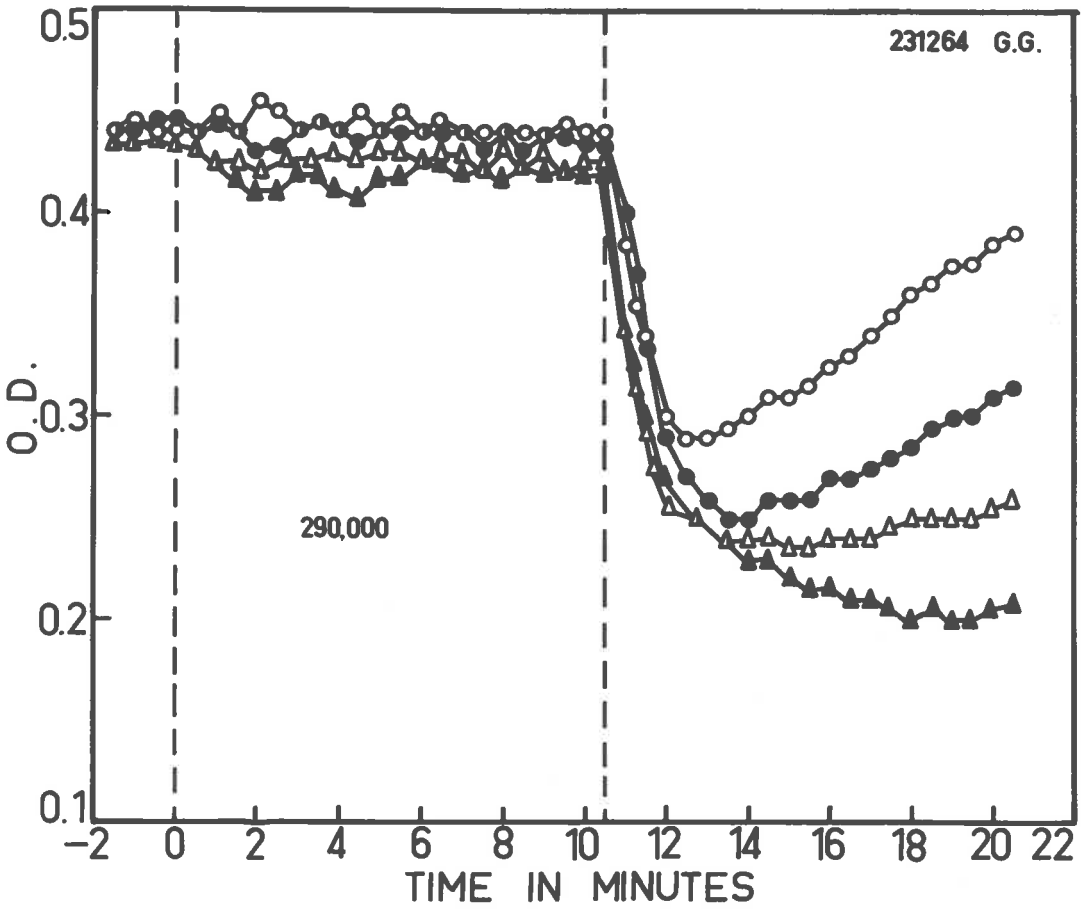


Fig. 51. Adrenaline potentiation of ATP-induced platelet aggregation. Adrenaline was added at 0 min., to give the following final concentrations: 2.5×10^{-8}M (●), 2.5×10^{-7}M (▲), and 2.5×10^{-6}M (▲). ATP (2.5×10^{-6}M) was added at 10.5 min. In the control experiment (○) saline was added at 0 min., followed by ATP (2.5×10^{-6}M). Adrenaline potentiation is evident. For a given concentration of ATP this potentiation increased with increasing concentrations of adrenaline up to a concentration of 2.5×10^{-6}M. An adrenaline concentration of 2.5×10^{-4}M (not graphed) produced no further fall in O.D.

differing concentrations of adrenaline on the aggregation produced by ATP at a constant final concentration of 2.5×10^{-6} M is shown. It can be seen that for a given nucleotide concentration, the degree of aggregation produced by the ATP-adrenaline combination increases with increasing adrenaline concentrations, reaching a maximum with an optimal final adrenaline concentration of 2.5×10^{-6} M. An increase in the final adrenaline concentration to 2.5×10^{-4} M (not graphed) resulted in no further aggregation. It is readily apparent that the enhanced aggregation is not simply a summation of the adrenaline and nucleotide activities.

An example of adrenaline potentiation of ADP clumping activity is shown in Figure 52. Here an inactive concentration of ADP (2.5×10^{-7} M) is transformed into one producing maximal aggregation by the prior addition of adrenaline at a final concentration of 2.5×10^{-5} M.

Using equimolar concentrations of both catecholamine and nucleotide, potentiation was greatest with adrenaline and least with dopamine (Fig. 53). Invariably the potentiation produced by adrenaline exceeded that of nor-adrenaline, while dopamine was the least active of the three. Potentiation of ADP or ATP clumping activity was demonstrable with low final catecholamine concentrations which alone were unable to produce aggregation. On one occasion, a final adrenaline concentration as low as 2.5×10^{-10} M was found to enhance ATP activity. The minimal final concentration of dopamine

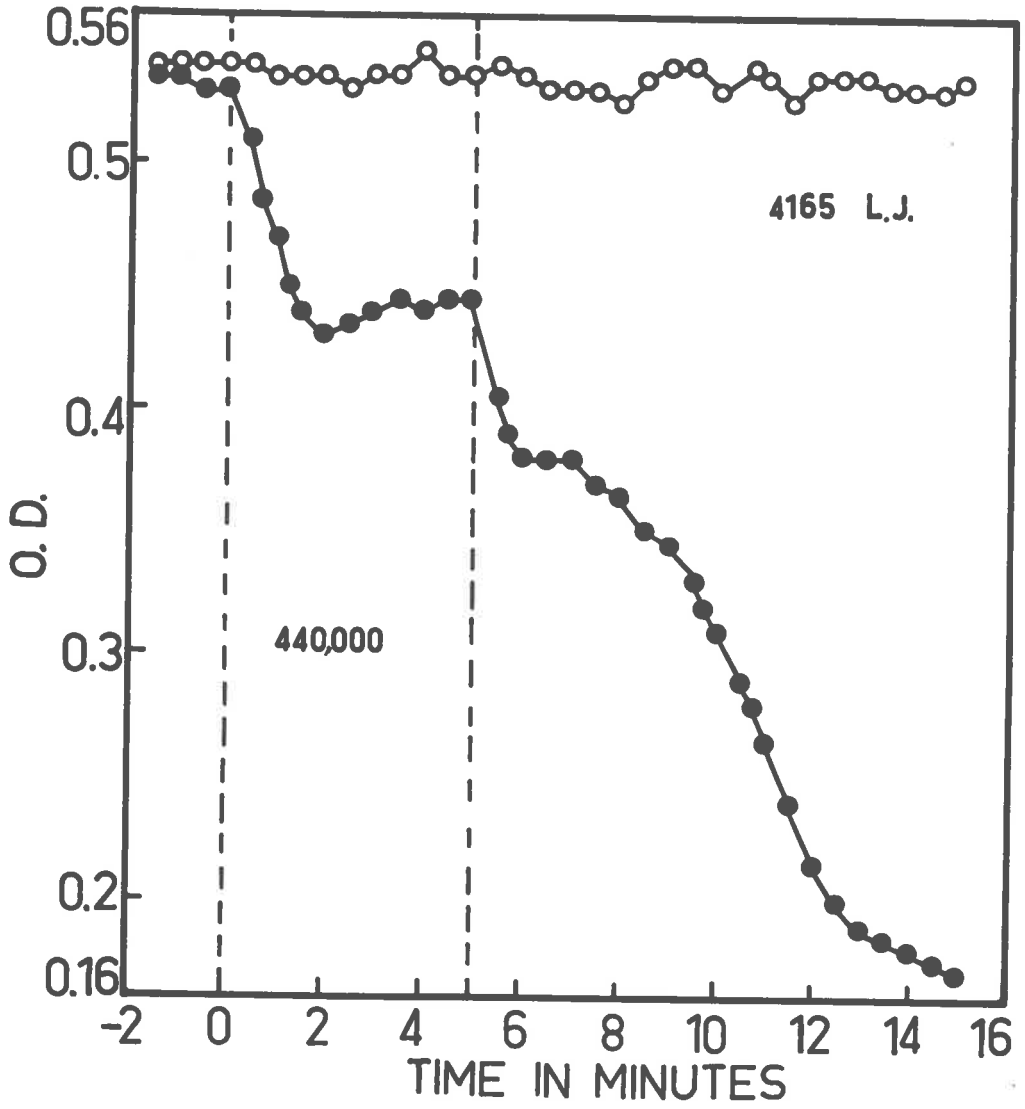


Fig. 52 . Adrenaline potentiation of ADP-induced platelet aggregation. Adrenaline (2.5×10^{-5}M) was added at 0 min., followed by ADP (2.5×10^{-7}M) at 5 min. (●). In the control experiment (○) saline replaced the adrenaline.

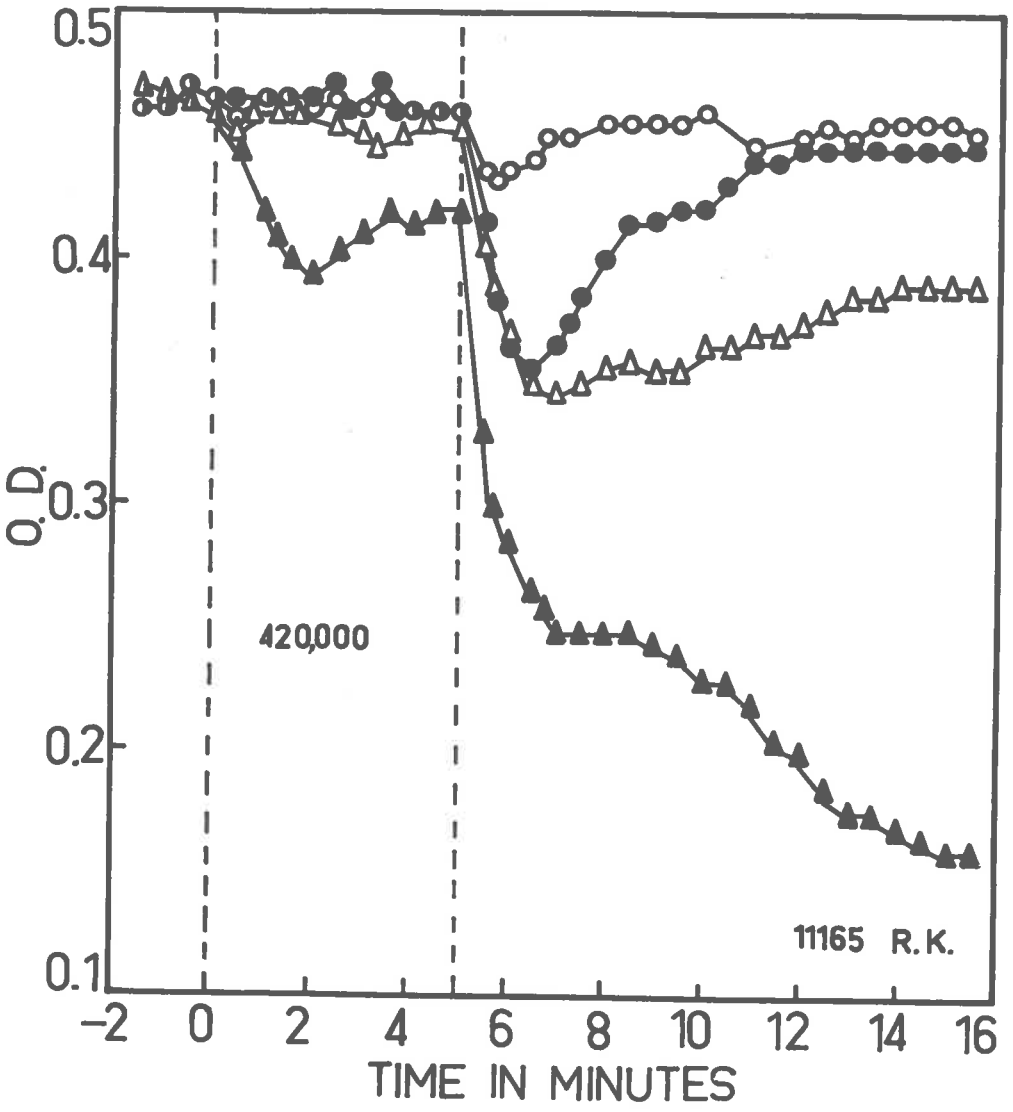


Fig. 53. Catecholamine potentiation of ADP-induced platelet aggregation. Equimolar concentrations (2.5×10^{-6}M) of adrenaline (▲), nor-adrenaline (△), and dopamine (●) were added at 0 min., followed by ADP (2.5×10^{-6}M) at 5 min. In the control experiment (○) saline replaced the catecholamine. Adrenaline produced the greatest potentiation and dopamine the least.

able to potentiate either ADP or ATP-induced platelet aggregation was 2.5×10^{-6} M. Nor-adrenaline was found to potentiate ATP and ADP clumping activity at concentrations of 2.5×10^{-6} M and above. The phenomenon of potentiation was not observed with those nucleotides which alone are incapable of producing platelet aggregation, namely adenosine monophosphate (AMP), adenosine, inosine triphosphate and inosine monophosphate. Nor did 5-hydroxytryptamine enhance nucleotide-induced aggregation.

Effect of N-ethyl maleimide on catecholamine potentiation

The sulphydryl inhibitor N-ethyl maleimide (NEM) is a powerful inhibitor of both nucleotide and catecholamine induced platelet aggregation. In a number of studies it was found that NEM could also prevent the catecholamine potentiation of nucleotide clumping activity. An example of this inhibition is shown in Figure 54, which also shows, in passing, the inhibitory effect of NEM both on ATP and also possibly on adrenaline clumping activity.

Prevention of adenosine and AMP inhibition of nucleotide-induced aggregation by the catecholamines

The platelet-clumping activity of ADP or ATP is either abolished or reduced by the prior addition of adenosine or AMP (see Table 2). In equimolar concentrations, adenosine is a more effective inhibitor than AMP. An example of AMP inhibition of ADP-induced aggregation is seen in Figure 55. When the saline of the control experiment (open circles) was substituted by

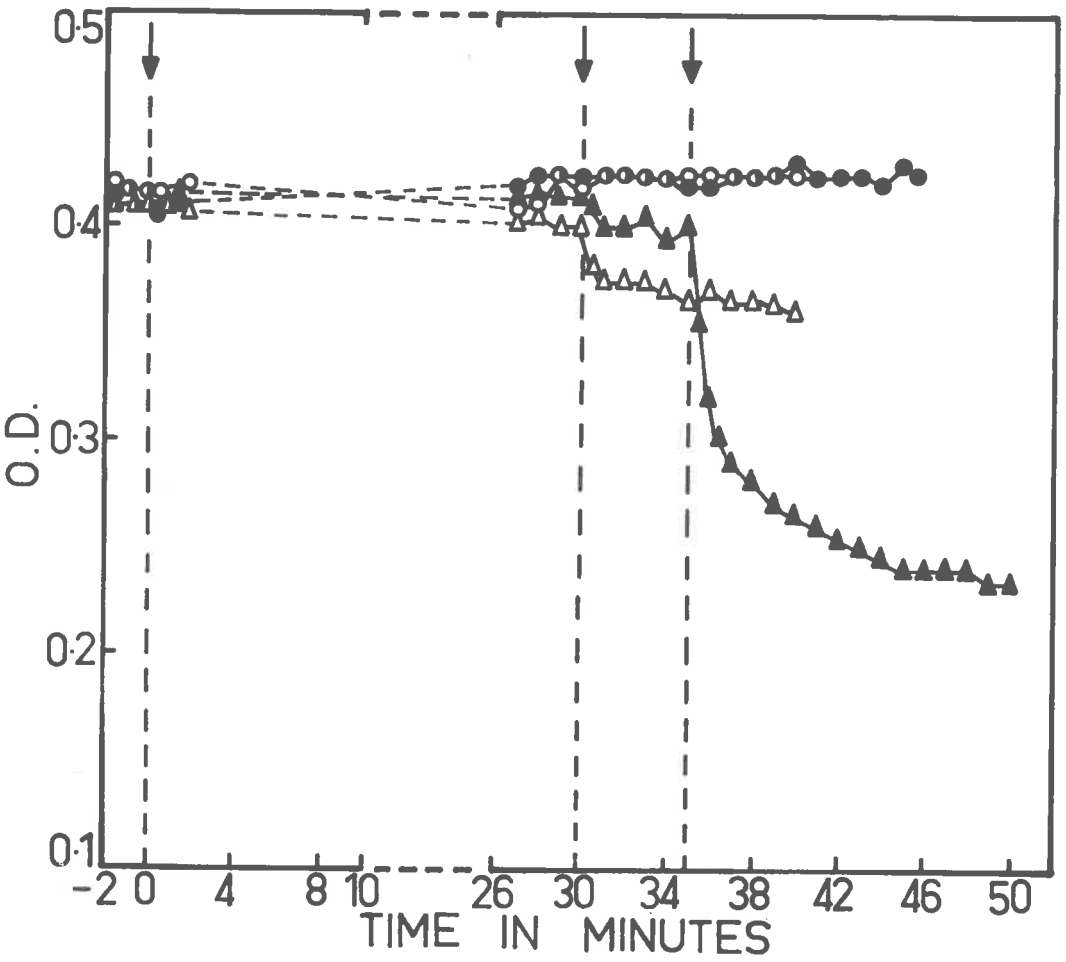


Fig. 54. The effect of NEM on ATP-induced aggregation and on the adrenaline-ATP system. NEM (2.5×10^{-4}M) was added at 0 min., and ATP (2.5×10^{-5}M) at 30 min. (O). In the appropriate control experiment (▲) saline replaced the NEM. When saline was added at 0 min., adrenaline (2.5×10^{-4}M) at 30 min., and ATP (2.5×10^{-5}M) at 35 min. (▲) a typical adrenaline-ATP potentiation response is seen. This response was completely inhibited when the saline in this experiment was replaced by NEM (●).

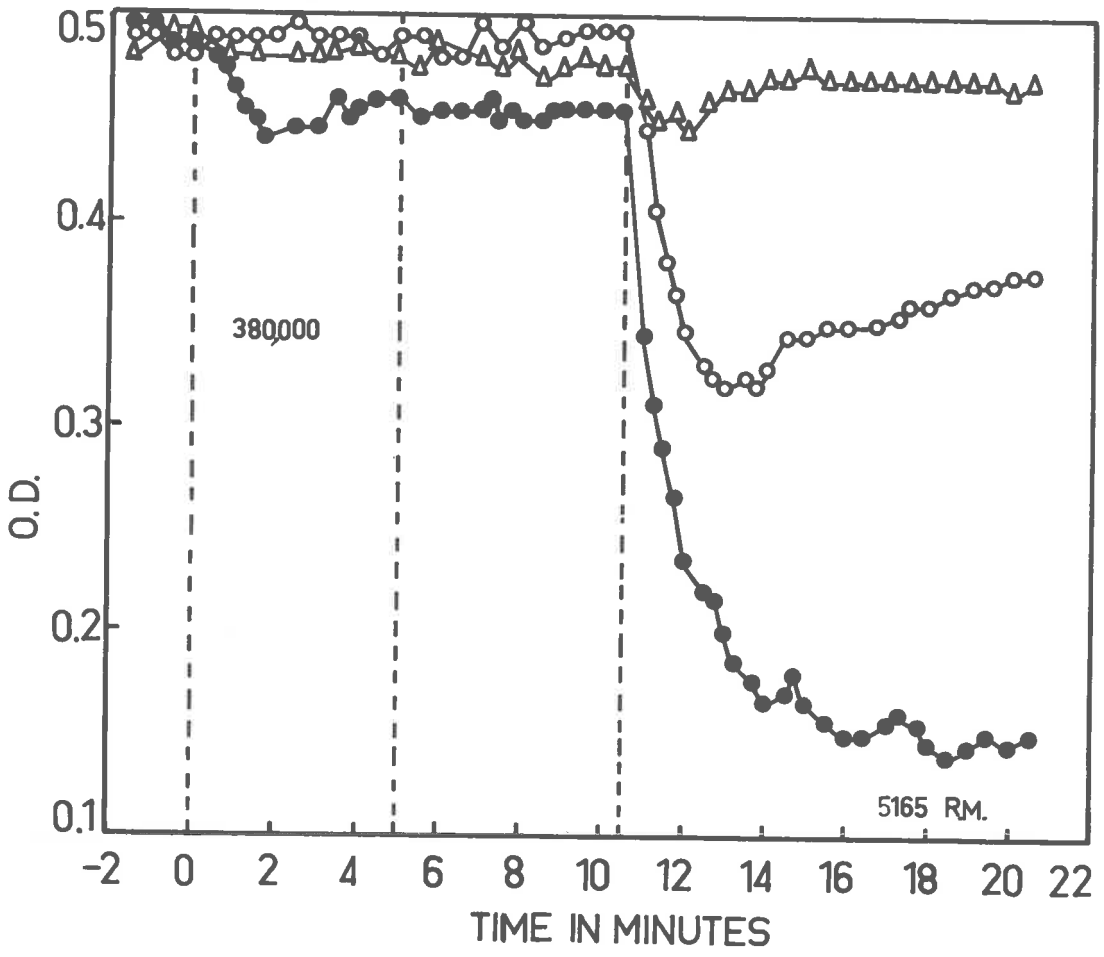


Fig. 55. AMP inhibition of ADP platelet clumping activity with prevention of this inhibition and subsequent potentiation by the prior addition of adrenaline. Saline was added 0 min., AMP (2.5×10^{-6}M) at 5 min., and ADP (2.5×10^{-6}M) at 10.5 min (Δ). When adrenaline (2.5×10^{-5}M) replaced saline at 0 min., and AMP was again added at 5 min., the effect of ADP (2.5×10^{-6}M) added at 10.5 min. (●) was enhanced, and the inhibitory effect of AMP was lost. In the control experiment (○) saline was added at 0 and 5 min., followed by ADP (2.5×10^{-6}M) at 10.5 min.

adrenaline at a final concentration of 2.5×10^{-5} M (closed circles) not only was the inhibitory effect of a subsequent addition of AMP lost, but the degree of aggregation produced by ADP (2.5×10^{-6} M final concentration) was strikingly enhanced. The effect of dopamine on adenosine inhibition of ADP-induced aggregation is shown in Figure 56. Nor-adrenaline also exhibited this ability to abolish the inhibition of AMP and adenosine on both ADP and ATP induced platelet clumping activity.

The platelet clumping activity of ADP or ATP is also inhibited by the prior addition of ADP or ATP. Figure 57 shows the inhibitory effect of ADP on ADP-induced aggregation. When ADP (2.5×10^{-6} M final concentration) was added to PRP 10.5 minutes after ADP at a final concentration of 2.5×10^{-7} M (open triangles), the response was partially inhibited by the first addition of ADP. However, when adrenaline (2.5×10^{-5} M final concentration) was added to the PRP 5 minutes after the first addition of ADP, the effect of ADP (2.5×10^{-6} M final concentration) added at 10.5 minutes was markedly enhanced (closed circles). The same effect with adrenaline was obtained when ATP was added before ADP, ATP before ATP or ADP before ATP. Figure 57 also shows that when the nucleotide is added to the PRP before the catecholamine, the potentiation phenomenon does not occur. The enhanced response only occurs when the nucleotide is added to the PRP after the catecholamine.

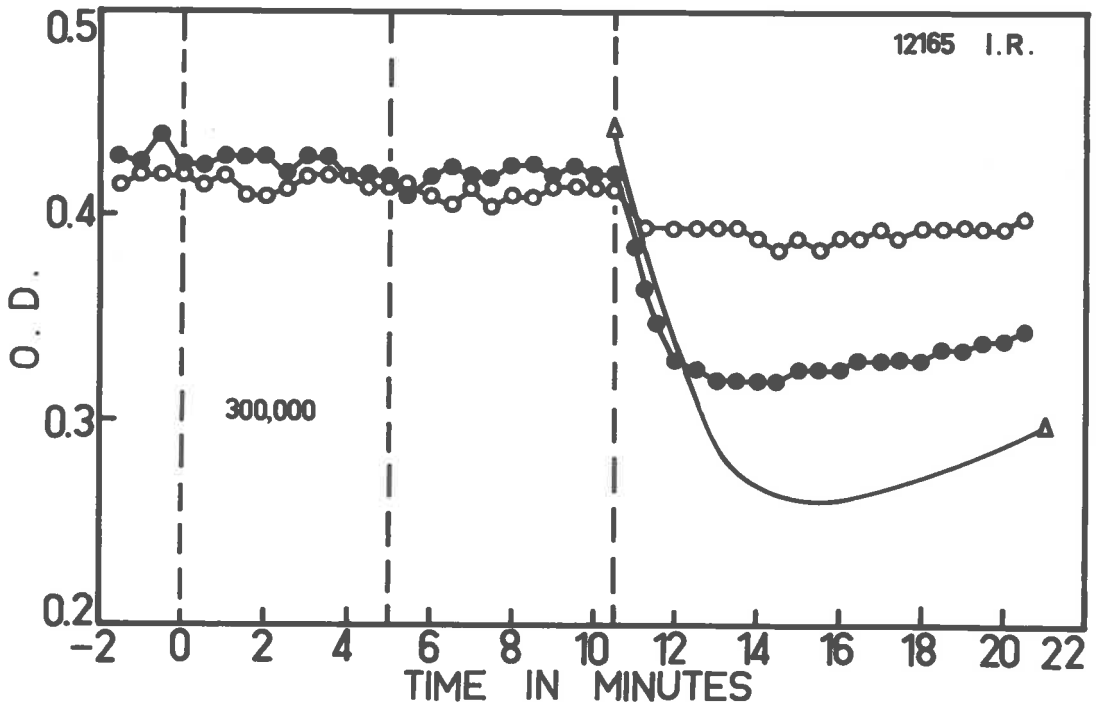


Fig. 56. Adenosine inhibition of ADP clumping activity with partial prevention of this inhibition by the prior addition of dopamine. Saline was added at 0 min., adenosine (2.5×10^{-6}M) at 5 min., and ADP (2.5×10^{-6}M) at 10.5 min. (O). When dopamine (2.5×10^{-5}M) replaced saline at 0 min., and adenosine was again added at 5 min., and ADP (2.5×10^{-6}M) at 10.5 min., the inhibitory effect of adenosine was diminished (●). In the control experiment (Δ) ADP (2.5×10^{-6}M) was added at 10.5 min.

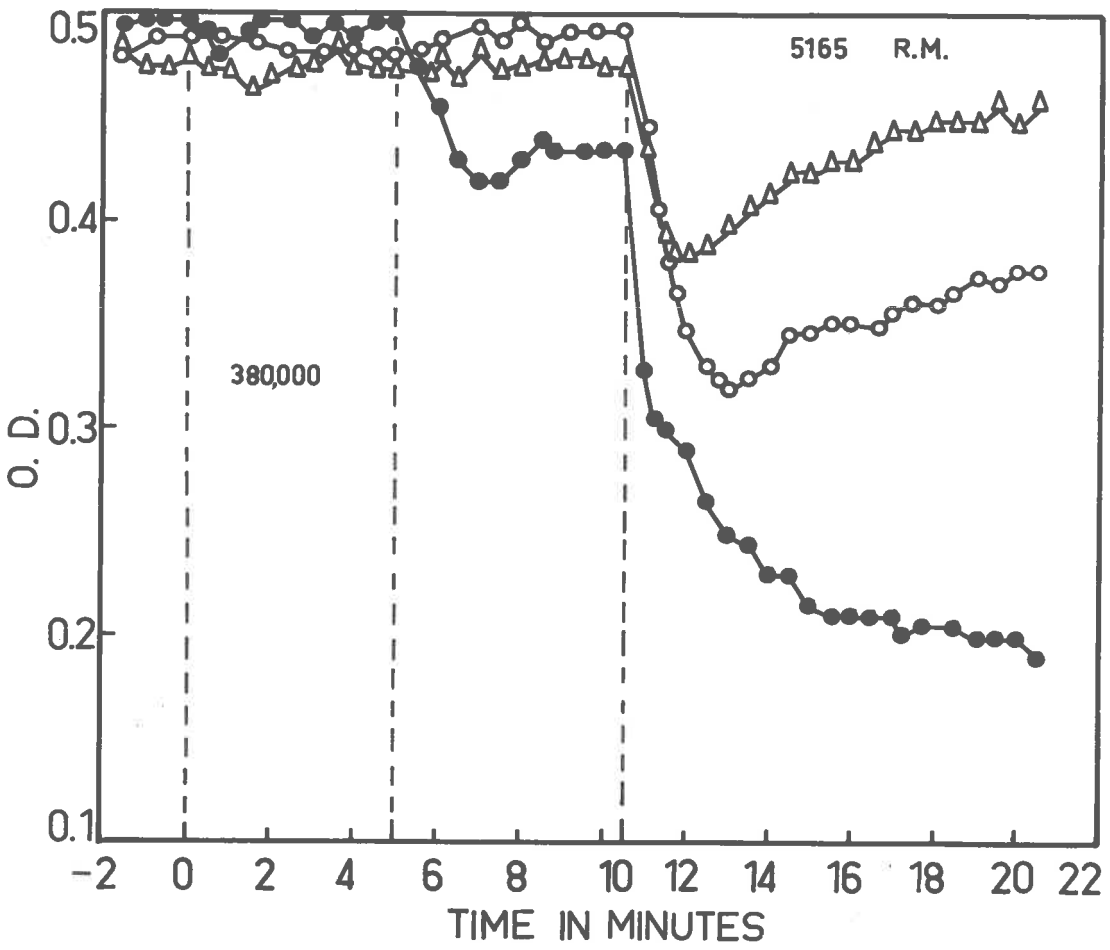


Fig. 57 . ADP inhibition of ADP-induced platelet aggregation with prevention of this inhibition and subsequent potentiation by adrenaline. In the control experiment (O) saline was added at 0 min., and ADP (2.5×10^{-6}M) at 10.5 min. When the saline was replaced by ADP (2.5×10^{-7}M), the response with ADP (2.5×10^{-6}M) added at 10.5 min., was less (Δ). However when ADP (2.5×10^{-7}M) was added at 0 min., and adrenaline (2.5×10^{-5}M) was added at 5 min., the response with ADP (2.5×10^{-6}M) added at 10.5 min. was markedly enhanced (●). Note that the addition of nucleotide to the plasma prior to the catecholamine does not produce potentiation.

Experiment 5: Inhibition and reversal of platelet aggregation by methyl xanthines

Considerable attention has been focused upon factors promoting aggregation, but compounds able to inhibit the process also merit attention, firstly because they may contribute further to an understanding of the mechanism of aggregation itself, and secondly because of their therapeutic possibilities. In this experiment, the inhibition and reversal of *in vitro* platelet aggregation by the methyl xanthines are described.

1. Materials and Methods

Three compounds were studied, namely caffeine citrate (1,3,7 - trimethylxanthine, May and Baker, batch D15), theobromine sodio-salicylate (3.7 - dimethylxanthine, Powers-Weightman-Rosengarten Corp., S.65.34, 42723), and aminophylline (1,3 - dimethylxanthine in ethylenediamine, C. H. Boehringer Sohn, batch B4687/292753). The methods employed were as described in experiment 4.

2. Results

Of the three compounds studied, neither theobromine nor aminophylline produced measurable platelet aggregation, but caffeine citrate appeared to have very slight clumping activity at a final concentration of 6×10^{-3} M, an example of which is seen in Figure 58.

At a final concentration of 6×10^{-3} M, all three xanthines under investigation consistently and completely inhibited the clumping activity of a subsequent and powerful clumping dose

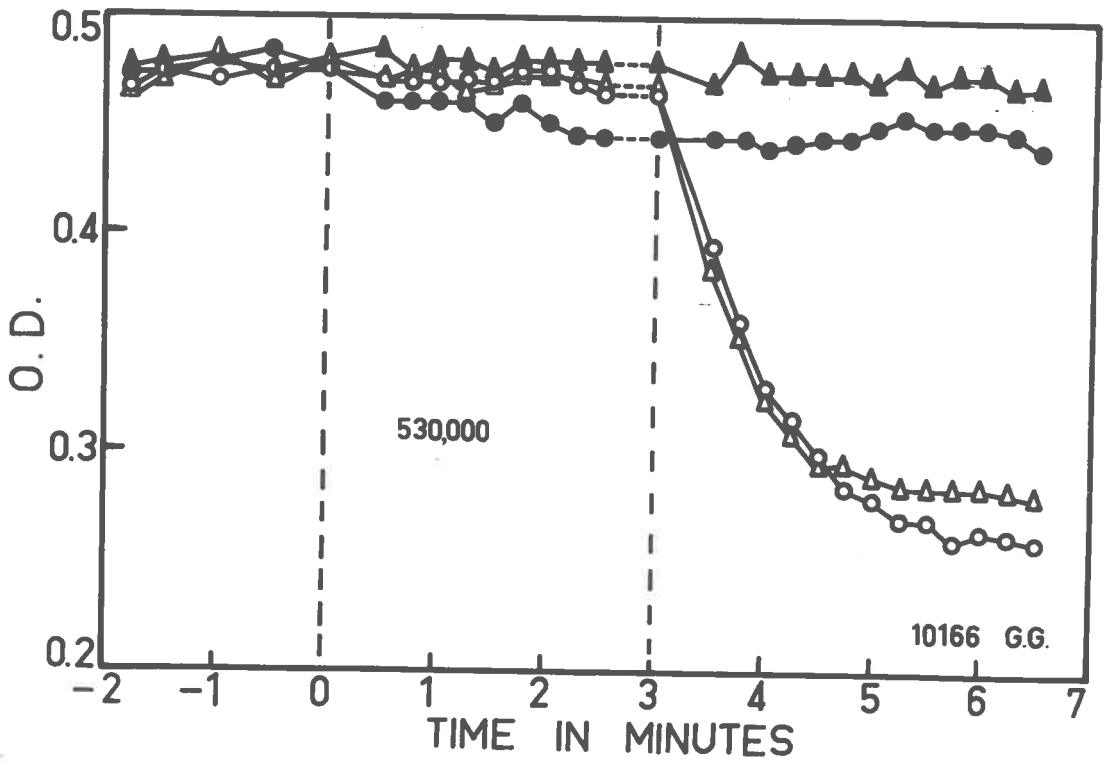


Fig. 58. Inhibition of ADP-induced aggregation by aminophylline and caffeine. Aminophylline (▲) or caffeine (●) at a concentration of 6×10^{-3}M was added at 0 min., and ADP (2.5×10^{-5}M) at 3 min. In the control experiment (○) the xanthine was substituted by an equal volume of 3.8% trisodium citrate. When the concentration of caffeine was reduced to 6×10^{-4}M (△) no inhibition was observed. Note the slight drop in optical density of the plasma after the addition of 6×10^{-3}M caffeine. (Platelet aggregation, however, was not confirmed microscopically.) Employing the method detailed by Lineweaver and Burk (J. Amer. Chem. Soc. 56, 658, 1934) this inhibition was found to be non-competitive in nature.

(2.5×10^{-5}M) of adenosine diphosphate (ADP). Examples of this inhibition are shown in Figure 58. This figure also shows that on reducing the final concentration of caffeine to 6×10^{-4}M, no inhibition of ADP-induced platelet aggregation was observed.

At a final concentration of 6×10^{-3}M, all three xanthine preparations studied were consistently found to reverse the marked platelet aggregation produced by 2.5×10^{-5}M ADP (Figs. 59 and 60). Reversal consistently commenced within 1 minute of the xanthine addition, and by 4 minutes was nearing completion. None of the three xanthines at a final concentration of 6×10^{-4}M produced any reversal of ADP-induced aggregation (Fig. 60).

Similarly, aminophylline at the same concentration (6×10^{-3}M) was found to reverse the aggregation produced by adenosine triphosphate (ATP) at a concentration of 2.5×10^{-5}M. Furthermore, it was demonstrated in experiment 4 that adrenaline enhances the clumping activity of ADP and ATP. Aminophylline (6×10^{-3}M) was also found to inhibit (Fig. 61) and reverse (Fig. 62) the aggregation produced by a combination of adrenaline and ATP or ADP. Figure 61 also shows that aggregation produced by adrenaline alone is also inhibited by aminophylline.

Several experiments were undertaken in an attempt to elucidate the mechanism whereby the methyl xanthines inhibit and reverse nucleotide-induced platelet aggregation. Neither inhibition nor reversal was found to be mediated by plasma pH changes produced

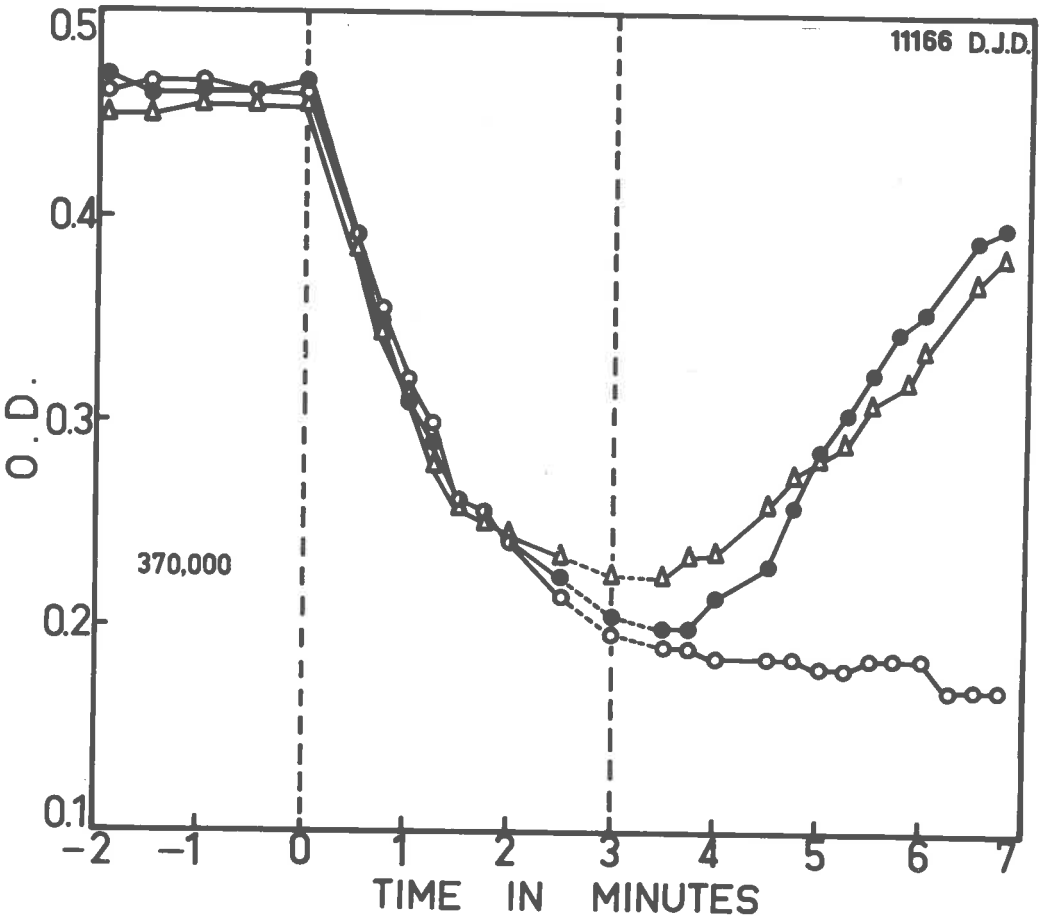


Fig. 59. Reversal of ADP-induced aggregation by aminophylline and caffeine. ADP (2.5×10^{-5}M) was added at 0 min., and aminophylline (Δ) or caffeine (●) at a concentration of 6×10^{-3}M was added at 3 min. Note that reversal commences within 1 min. and is nearing completion 4 min. after the addition of either methyl xanthine. In the control experiment (O) the xanthine was substituted by an equal volume of 3.8% trisodium citrate.

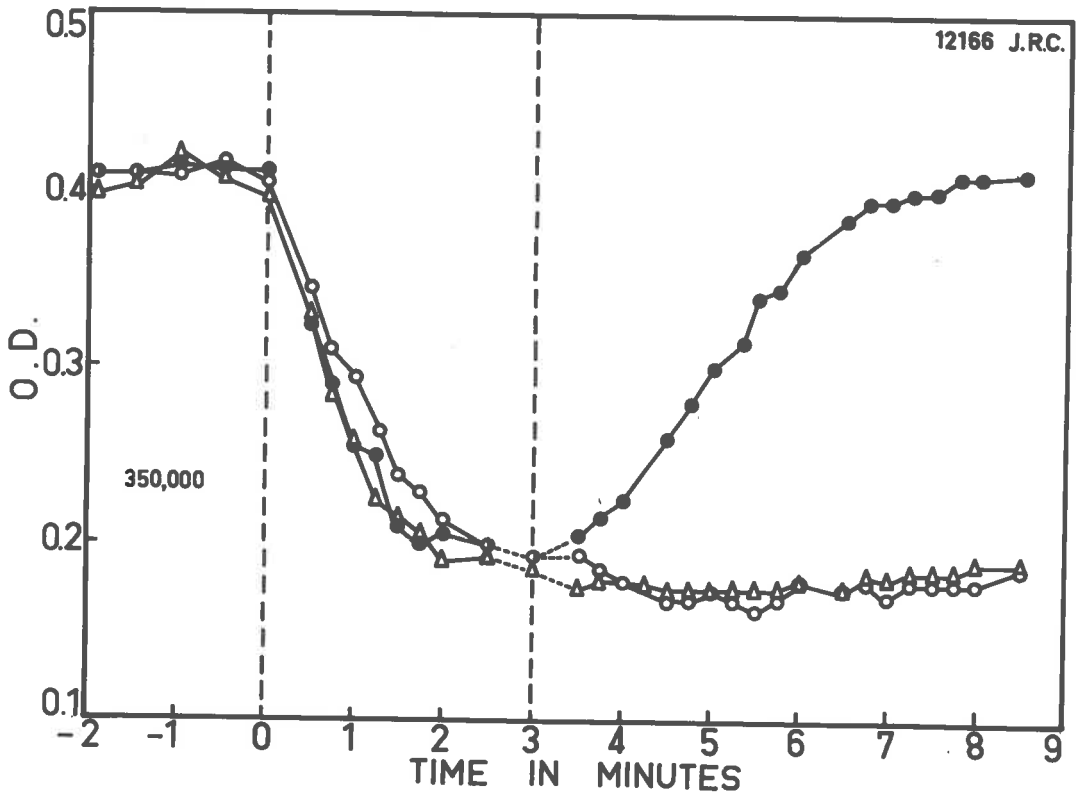


Fig. 60. Reversal of ADP-induced aggregation by theobromine. ADP (2.5×10^{-5}M) was added at 0 min., and theobromine (6×10^{-3} M) at 3 min. in the test experiment (●). In the control experiment (O) the xanthine was substituted by an equal volume of 3.8% trisodium citrate. When the concentration of theobromine was reduced to 6×10^{-4}M (Δ) no inhibition was observed. Additional experiments showed that sodium salicylate and ethylenediamine, components of two of the methyl xanthines examined, had no influence on platelet aggregation.

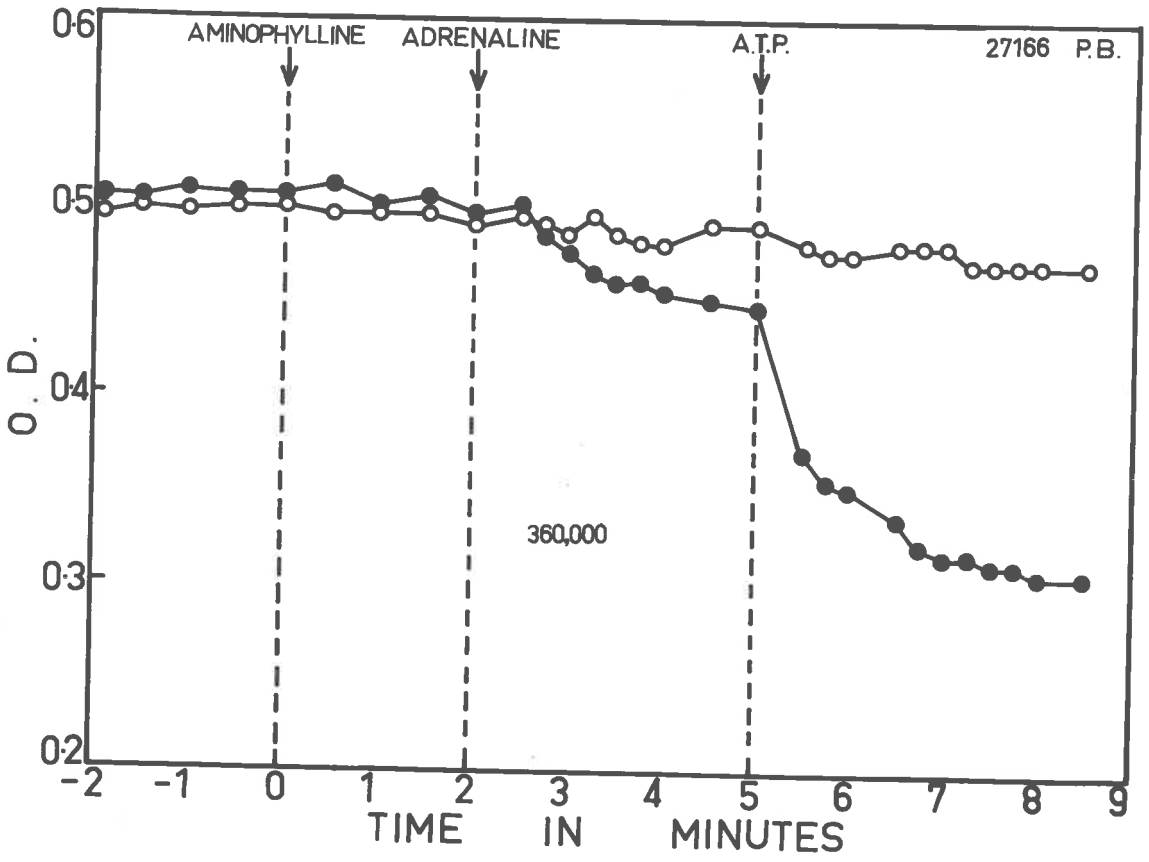


Fig. 61. Inhibition of adrenaline-induced aggregation and the adrenaline-ATP response by aminophylline. Aminophylline ($6x10^{-3}M$) was added at 0 min., adrenaline ($2.5x10^{-5}M$) at 2 min., and ATP ($2.5x10^{-5}M$) at 5 min. (O). In the control experiment saline was added at 0 min., adrenaline ($2.5x10^{-5}M$) at 2 min., and ATP ($2.5x10^{-5}M$) at 5 min. (●).

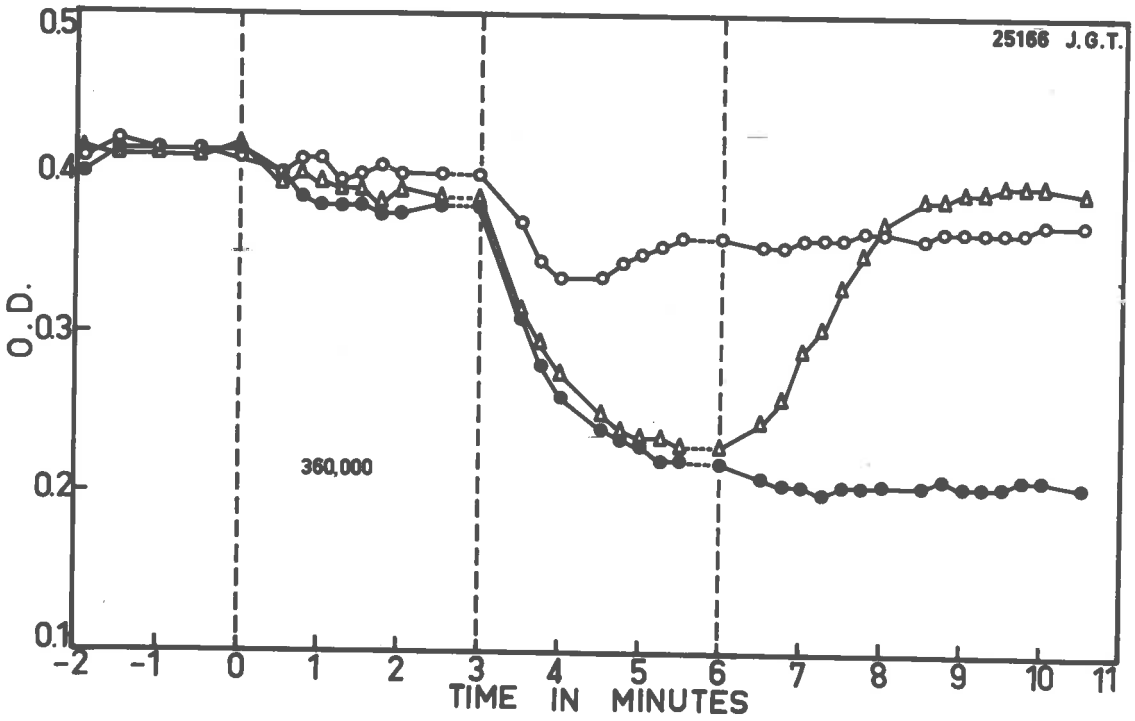


Fig. 62. Reversal of the adrenaline-ADP system by aminophylline. In the test experiment (Δ) adrenaline (2.5×10^{-5}M) was added at 0 min., ADP (2.5×10^{-6}M) at 3 min., and aminophylline (6×10^{-3}M) at 6 min. In the control experiment (●) the xanthine was substituted by saline. The adrenaline-ADP effect is evident when compared with the other experiment (○) in which saline was added at 0 min., ADP (2.5×10^{-6}M) at 3 min., and saline again at 6 min.

by the xanthines. Moreover, the inhibitory effect of these xanthines was found to be non-competitive.

Both cysteine and reduced glutathione in adequate concentrations are able to overcome the inhibitory and reversal effects of the sulphydryl inhibitor, N-ethyl maleimide, on ADP-induced platelet aggregation. To exclude the possibility that the methyl xanthine action might be mediated through a similar effect on platelet SH-groups, the effects of cysteine and reduced glutathione on methyl xanthine inhibition and reversal were studied. Neither cysteine in a final concentration of 6×10^{-3} M, nor reduced glutathione (2.5×10^{-4} M) had any influence on the inhibition or reversal of ADP-induced platelet aggregation by the methyl xanthines.

EDTA (3%) can inhibit and reverse the platelet clumping produced by ADP, both the inhibition and reversal being abolished by the addition of excess calcium chloride. The latter, however, at a final concentration of 2.2×10^{-3} M did not influence the caffeine inhibition of ADP-induced platelet aggregation, indicating that the xanthine effect is not mediated through chelation of calcium ions.

Experiment 6: Fatty acids and in vitro platelet aggregation

Considerable epidemiological evidence suggests that abnormal lipid metabolism and a tendency to thrombosis may be related. Among the plasma lipids that may be altered by changes in dietary fat are the unesterified fatty acids. Long

chain fatty acids have been shown to promote thrombus formation in an in vitro system (Connor and Poole, 1961) and also when injected intravenously into dogs (Connor, Hoak and Warner (1963) or into the vessels of the bat's wing (Soloff and Wiedeman, 1963). Haslam (1964) found that behenate, stearate and other saturated fatty acids produced aggregation of washed human platelets, and Kerr et al. (1965) showed that various phospholipid-fatty acid solutions were able to cause platelet aggregation in vitro.

In this experiment the effects of the sodium salts of saturated and unsaturated fatty acids on human platelet-rich plasma are reported. The platelets were not subjected to washing as this is considered to damage these cells.

1. Materials and Methods

Sodium salts of the fatty acids, palmitic, stearic, oleic, linolenic and linoleic (Calbiochem, A grade) were prepared by the addition of an appropriate volume of 0.1 M NaOH and diluted to give a concentration of 2.5×10^{-3} M. These were heated in a water bath at 60° prior to use. The methods employed were as described in experiment 4.

2. Results

In final concentrations of 2.5×10^{-5} or 0.83×10^{-4} M, none of the 5 fatty acids tested showed any measurable effect on platelet aggregation. Calcium chloride in a final concentration of 1.1×10^{-4} M did not modify the response. Further, none of

the 5 fatty acids at a final concentration of 0.83×10^{-4} M showed any effect on the platelet aggregation induced by ADP (2.5×10^{-6} M), when the latter was added to the cuvette 5 or 10 minutes after the addition of the fatty acid. In control experiments the fatty acid was replaced by an equal volume of 0.9% saline.

In low concentrations the catecholamines, adrenaline, noradrenaline and dopamine have previously been shown to enhance the platelet clumping activity of a subsequent addition of adenosine triphosphate (ATP) or ADP (experiment 4). The effect of the sodium salts of 5 fatty acids on this adrenaline-ATP response were examined. In each experiment the fatty acid in a final concentration of 0.83×10^{-4} M preceded the adrenaline (2.5×10^{-6} M final concentration) by an interval of 5 minutes, and the ATP (2.5×10^{-6} M final concentration) was added 5.5 minutes later. As the degree of platelet aggregation for a given concentration of nucleotide diminishes with the passage of time after blood collection, each experiment was accompanied by an adequate control. In each control the fatty acid was substituted by the addition of 0.1 ml. of 0.9% saline.

Figures 63 and 64 show the results of typical experiments in which the adrenaline-ATP response is visibly enhanced by sodium stearate. Stearic acid was the only fatty acid found to enhance the platelet clumping activity of this system, palmitate, oleate, linoleate and linolenate being consistently without effect. The

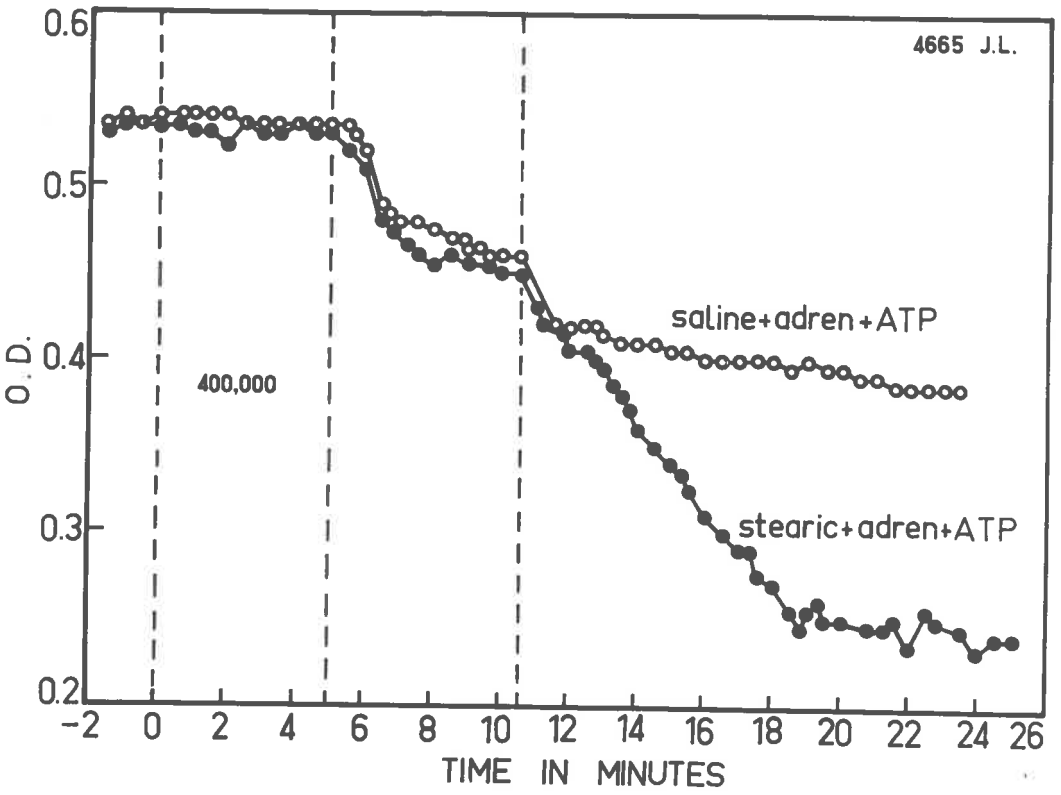


Fig. 63. The effect of sodium stearate on the adrenaline-ATP system. Sodium stearate (0.83×10^{-4}M) was added at 0 min., adrenaline (2.5×10^{-6}M) at 5 min., and ATP (2.5×10^{-6}M) at 10.5 min. in the test experiment (●). In the control experiment (○) saline replaced the fatty acid.

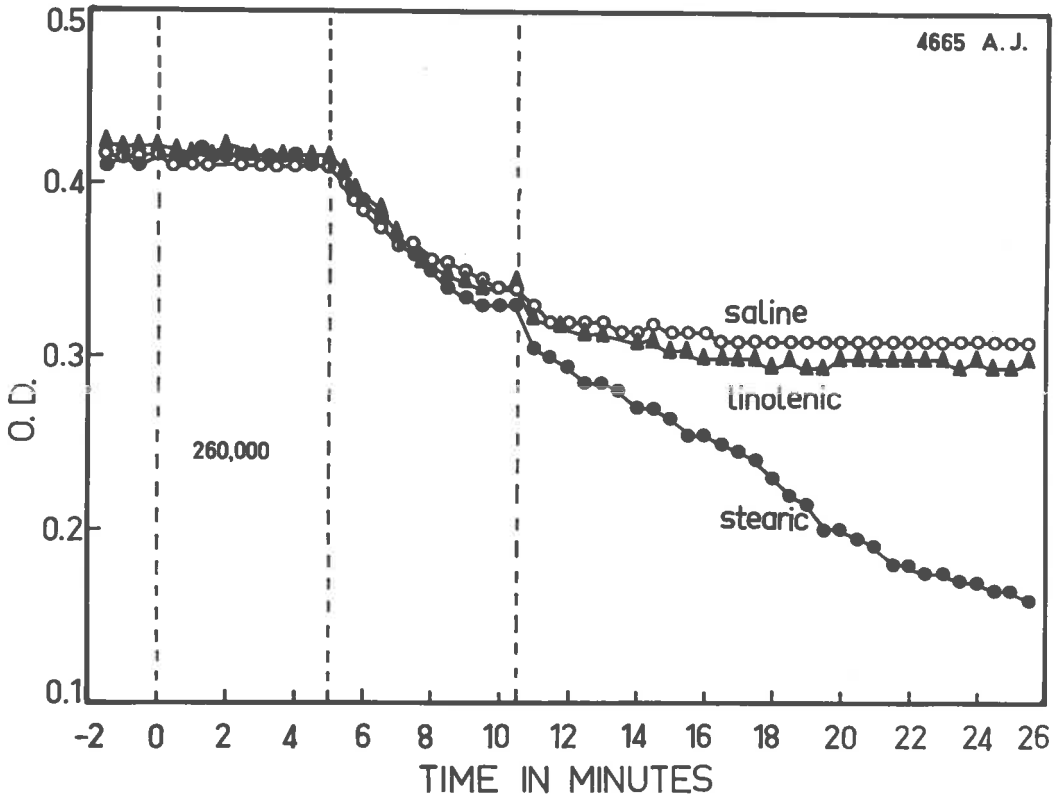


Fig. 64. The effect of sodium stearate on the adrenaline-ATP system. Sodium stearate (0.83×10^{-4}M) was added at 0 min., adrenaline (2.5×10^{-6}M) at 5 min., and ATP (2.5×10^{-6}M) at 10.5 min. in the test experiment (●). In the control experiment (○) saline replaced the fatty acid. Sodium linolenate (▲) did not modify the adrenaline-ATP response.

TABLE 9

Fatty acid tested	Number of expts.	Mean fall in plasma optical density below control level (%)							
		Time after ATP (min.)							
		1.5	3.5	5.5	7.5	9.5	11.5	13.5	14.5
Palmitic C16:0	5	-2.4	-1.8	-0.2	-0.2	-0.7	-0.7	-1.1	-0.1
Stearic C18:0	13	0.9	4.4	7.9	11.6	15.1	17.3	19.5	20.7
Oleic C18:1	5	1.9	4.0	4.6	2.7	2.3	1.3	0.9	2.3
Linoleic C18:2	4	2.7	0.9	2.2	1.1	0.4	0.7	0.4	0.5
Linolenic C18:3	4	0.6	0.6	2.2	1.9	2.4	1.0	1.0	2.8

The effect of the sodium salts of 5 fatty acids on platelet aggregation in the adrenalin-ATP system. The mean fall (%) in plasma optical density below the control levels is tabulated for varying times after the addition of ATP (see text).

results of 31 of these experiments are detailed in Table 9 where the mean percentage fall in plasma optical density (OD) below the control level is shown for the 5 fatty acids at varying times after the addition of ATP. The difference between the OD of plasma at 10 minutes (Fig. 63) and the OD of platelet-free plasma was taken as 100%. Percentage change in plasma optical density was calculated for test and control experiments, and the percentage fall below the control level was derived by difference. It is at once apparent that palmitate, oleate, linoleate and linolenate did not enhance or inhibit the platelet aggregation produced by the adrenaline-ATP system. Stearate, on the other hand, clearly enhanced aggregation, the mean fall in plasma OD below the control level increasing with time to a maximum (20.7%) 14.5 minutes after ATP was added.

In several experiments it seemed that adrenaline clumping activity was itself enhanced by sodium stearate (Fig. 65).

The significance of these observations with stearic acid await further experiment. The non-esterfied fatty acids are carried in the blood bound to albumin, and in this form their properties are probably modified. If a disturbance in the normal binding mechanism can occur, then the effects of stearic acid observed in this experiment may be relevant to the in vivo situation. The effects of albumin bound fatty acids on in vitro platelet aggregation are being investigated in this laboratory.

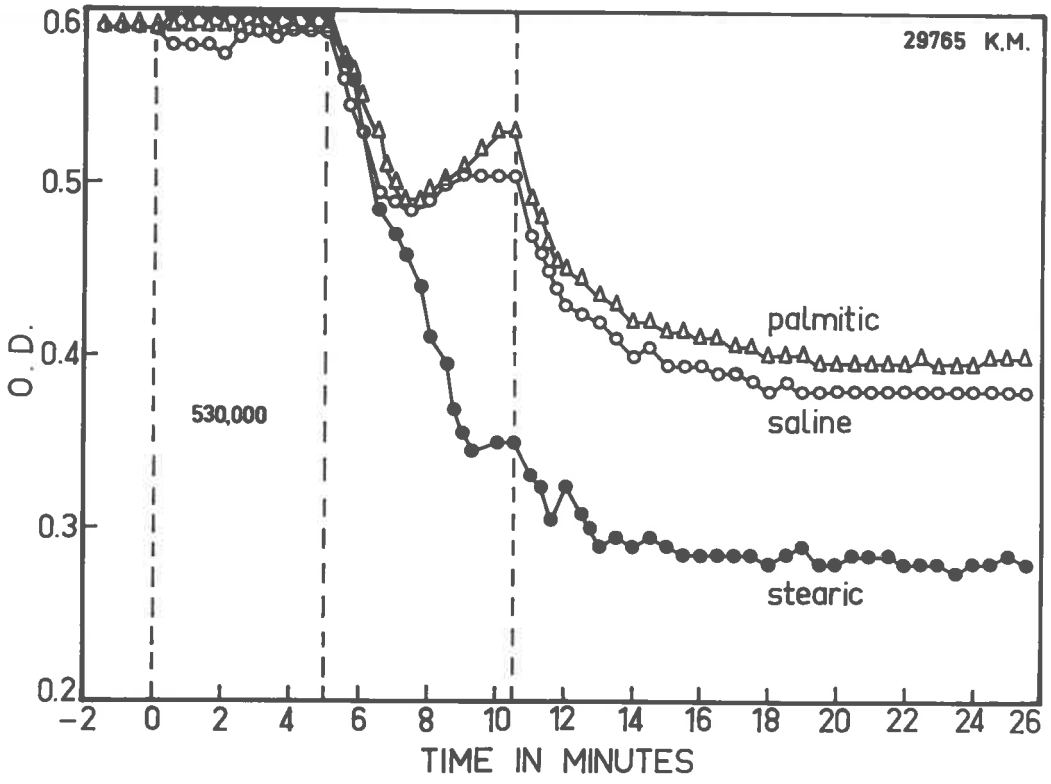


Fig. 65. The effect of sodium stearate on the adrenaline-ATP system. In the test experiment (◉) sodium stearate (0.83×10^{-4} M) was added at 0 min., adrenaline (2.5×10^{-6}M) at 5 min., and ATP (2.5×10^{-6}M) at 10.5 min. In the control experiment (O) saline replaced the fatty acid. Sodium palmitate (Δ) did not modify the adrenaline-ATP response.

Summary of Part III

The effects of the three catecholamines, adrenaline, nor-adrenaline and dopamine were studied using a spectrophotometric technique for the quantitative measurement of *in vitro* platelet aggregation.

Adrenaline and nor-adrenaline produced platelet aggregation, but dopamine was inactive. The degree of aggregation for a given concentration of adrenaline showed considerable variation between subjects and in the one subject on different days. Adrenaline induced aggregation was inhibited by phentolamine which also inhibited the activity of ADP and ATP.

All three catecholamines markedly enhanced the degree of aggregation produced by a subsequent addition of ADP or ATP, and abolished the inhibitory action of adenosine, AMP, ATP and ADP on nucleotide induced aggregation. This phenomenon of catecholamine potentiation was not merely a summation of activities, the effect being much greater than this. Adrenaline produced the greatest and dopamine the least potentiation. Potentiation was evident with low final concentrations of nucleotide or catecholamine which alone were unable to produce measurable aggregation. The potentiation effect was not seen when the catecholamine was added to plasma after the nucleotide, only before. Catecholamine potentiation was further enhanced by sodium stearate but not by the sodium salts of oleic, palmitic,

linolenic or linoleic acid. The sulphydryl inhibitor, N-ethyl maleimide abolished the phenomenon, and various methyl xanthines were found to prevent and reverse the enhanced aggregation produced by the catecholamine-nucleotide combination.

The three methyl xanthines, caffeine, theobromine, and aminophylline also inhibited and reversed platelet aggregation induced by ADP in vitro. Inhibition appeared to be non-competitive and the effects of the methyl xanthines were not mediated through inhibition of platelet sulphydryl groups, chelation of calcium ions, or plasma pH change.

Discussion of Part III

Catecholamines, methyl xanthines and platelet aggregation

It has been shown (Bull and Zucker, 1965) that ADP or thrombin in aggregating concentrations produce a rapid increase in platelet volume, adrenaline being without effect. Employing a spectrophotometric technique, in which a reduced tracing amplitude reflects a change in platelet shape, O'Brien and Heywood (1966) demonstrated that ADP, 5-hydroxytryptamine, thrombin, and collagen all cause platelets to become more symmetrical immediately preceding the onset of aggregation. Again, no detectable change in shape resulted from the addition of adrenaline. This difference between adrenaline and the other aggregating agents such as ADP suggests either that the shape-volume changes are not essential for platelet aggregation or that

the aggregation produced by adrenaline is mediated through a mechanism different to that which operates for the other aggregating agents. In this regard it is of interest that EDTA, which inhibits ADP-induced aggregation, itself causes a change in shape (O'Brien and Heywood, 1966) and an increase in volume (Bull and Zucker, 1965) but does not cause aggregation. Cations appear to be essential, however, for ADP-induced aggregation and the absence of aggregation is probably due to the chelating properties of EDTA. The author considers that these changes in shape and volume result from product inhibition of a platelet membrane ATP-ase (transport ATP-ase) associated with the sodium pump mechanism. The failure of adrenaline to produce a measurable change in shape or volume is consistent with this view, for at present adrenaline has not been shown to play a significant role in sodium transport.

Whatever the intermediate biochemical sequences, it is probable that the final common pathway for platelet aggregation must involve a reduction in net surface charge or charge density (charge per unit surface area), and any explanation for catecholamine or nucleotide action should take this into consideration. This belief receives support from the work of Hampton and Mitchell (1966c) who have shown that with aggregating concentrations of ADP, ATP, nor-adrenaline, thrombin and collagen platelet electrophoretic mobility is reduced.

Jones (1966) has just recently proposed an interesting

contractile protein model for cell adhesion, which in the case of platelets depends on the observation that they, in common with other cells contain an actomyosin-like protein with ATP-ase activity (Bettex-Galland and Luscher, 1959). This model is dependent on the fact that proteins posses both negatively-charged carboxyl groups and positively charged side chains. Folding of this surface protein layer due to contraction of an actomyosin-like protein either at or just below the surface, results in the neutralisation of positively charged groups by adjacent carboxyl groups with a consequent increase in the negative charge per unit surface area. Relaxation of the contractile system reduces the negative charge per unit surface area and increases adhesiveness, as adhesion and surface charge are inversely related.

Salzman and his colleagues (1966b) have suggested that an active energy-supplying metabolic process is necessary to maintain platelets in a non-adhesive state. If the contractile model for cell adhesion is valid for platelets, this energy would be expended in maintaining the platelet actomyosin-like protein in a state of contraction, thus rendering the platelets less or non-adhesive. ADP-induced aggregation is then readily explained on the basis of product inhibition of the reaction $ATP \xrightarrow{\text{ATP-ase}} ADP$.

It is indeed difficult, because of a paucity of basic biochemical data, to explain the various effects of the catecholamines on platelet aggregation. However, the following mechanism albeit

speculative is proposed by the author. It is based on a series of experiments involving the enzyme adenyl cyclase, which has led to the development of a generalised picture of hormone action. These experiments have been conducted by Sutherland and his co-workers in the last decade, and their views are summarised in a recent article (Sutherland, Oye and Butcher, 1965) in which a two-messenger mechanism by which several hormones act on effector cells is described. The hormone (the first messenger) interacts with a component of the cell membrane (adenyl cyclase) producing an accumulation of a mediator, cyclic, 3', 5'-AMP (the second messenger), which then acts upon components of the effector cell.

In heart and liver preparations it has been shown (Murad et al., 1962) that adrenaline and nor-adrenaline enhance the activity of an enzyme, adenyl cyclase, which, in the presence of ATP results in the formation of cyclic 3', 5'-phosphate (cyclic AMP). The latter greatly stimulates the activity of the enzyme dephosphophosphorylase kinase converting inactive phosphorylase to an active form (Fig. 66). Not only does adrenaline enhance the formation of cyclic AMP from ATP, but it stimulates the release from the cell of cyclic AMP against a concentration gradient (Davoren and Sutherland, 1963). Thus adrenaline could theoretically deplete a platelet of its energy store with consequent failure of the contractile system so making the cell more adhesive. In this regard, the ability of the methyl xanthines to inhibit and reverse the aggregation produced by ADP is of particular

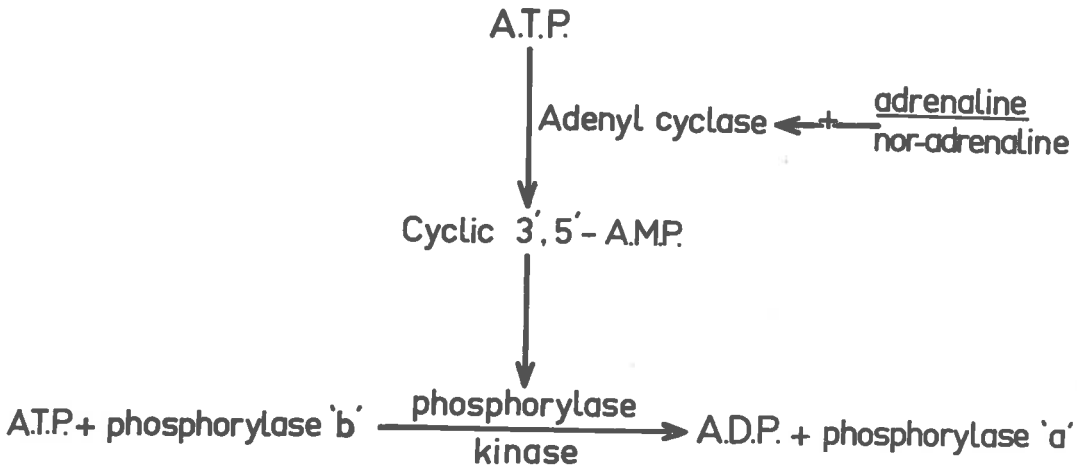


Fig. 66. A diagrammatic presentation of the formation and fate of cyclic 3', 5' - A. M. P., showing the involvement of the catecholamines, adrenaline and nor-adrenaline in this system.

interest. Like adrenaline, they cause an increase in the cell concentration of cyclic AMP by inhibiting its conversion to AMP, but differ by preventing the cellular release of cyclic AMP (Davoren and Sutherland, 1963). It is suggested that the formation and fate of cyclic AMP could modulate the energy supplying system necessary for contraction of the platelet actomyosin system and that the opposite effects of the methyl xanthines and catecholamines on platelet aggregation are related to their differing effects on cyclic nucleotide metabolism. It is not without interest that insulin has also been found in rat adipose tissue to lower intracellular levels of cyclic AMP (Butcher et al., 1966). In order to determine if this hypothetical mechanism is of any significance in the action of catecholamines in platelet aggregation, the presence or absence of platelet adenyl cyclase activity, and the role of cyclic AMP in platelet metabolism need urgent clarification.

All experiments to date indicate that all, or almost all, adenyl cyclase is in cell membranes (Davoren and Sutherland, 1963a) and it is therefore unnecessary for the catecholamines to enter the cell. They could exert their action by influencing the production of cyclic AMP at the membrane and the nucleotide could then, in turn, influence metabolism within the interior of the cell. In a personal communication, Sutherland stated that adenyl cyclase had been found in the white blood cells of all species studied, including man. In several instances he had been unable to find

any cyclase in human erythrocytes; assays on platelets are intended.

The evidence from several ultrastructural studies is consistent with the above proposals. White and Krivit (1965) found adenosine triphosphatase activity in the membranes of platelets by combined histochemical and electron microscopic techniques. Microtubules have been demonstrated in the hyaloplasm at the borders of platelets by three groups of workers (Sandborn, LeBuis and Bois, 1966; Sixma and Molenaar, 1966; Behnke, 1966) and it has been suggested that these may be the morphological substrates of the contractile proteins known to be present in platelets.

O'Brien (1963b) showed that phentolamine, a specific inhibitor of adrenaline, caused prolongation of the bleeding time. This observation tempts one to speculate whether adrenaline may normally be involved in platelet adhesion and aggregation in the haemostatic process, and therefore possibly in the development of thrombosis. Shimamota (1963) has suggested that Cannon's "emergency reaction" should be expanded to include the "platelet sticking reaction" a name which he has given to describe the adhesiveness of platelets to vascular endothelium and which can eventuate in the aggregation of platelets and the formation of white thrombi. This reaction was demonstrated in experiments on animals injected with adrenaline. Furthermore, stearic acid and other fats of animal origin produced this reaction.

Inhibition of nucleotide induce daggregation by adenosine and

AMP is well known. In experiment 4 it was shown that this action is abolished by the prior addition of adrenaline, nor-adrenaline or dopamine. The fact that the inhibiting mechanism is so effectively abolished by the catecholamines is of considerable importance, particularly as adenosine and its analogues are being investigated as agents which might prevent aggregation and therefore be used therapeutically in the prevention or treatment of thrombosis. In view of the catecholamine action, this avenue does not appear fruitful.

The ability of catecholamines at low concentrations to produce platelet aggregation, to enhance nucleotide-induced aggregation, and to abolish the inhibitory effects of adenosine and AMP are potentially of considerable importance, and suggest that catecholamines could be important in thrombosis. Perhaps the alleged relationship between stress and coronary disease might be explained on this basis. There are also many other observations such as the effect of cigarette smoking on catecholamine excretion and free fatty acid mobilisation (Kershbaum, et al., 1966), that are relevant to this discussion. However, until more is known of the effects of catecholamines in vivo these various observations cannot be adequately evaluated.

Sodium salts of fatty acids and platelet aggregation

Haslam (1964) has commented upon the problem of solubility

of the sodium salts of fatty acids. It is known that they form micelles and that the opalescence of sodium stearate solutions indicates the presence of micelles of polymolecular dimensions (White, Handler, Smith and Stetten, 1954). Solubility of these salts is said to decrease with increasing length of the hydrocarbon chain. Connor and Poole (1961) questioned whether the presence of polymolecular micelles might affect *in vitro* thrombus formation. Of the fatty acid salts tested, stearate, the least soluble, was the only one found to enhance platelet aggregation in the adrenaline-ATP system, but whether this effect is dependent upon micelle size remains unknown. This problem of solubility has recently been approached by the preparation of phospholipid sols (Kerr *et al.*, 1965).

It is of interest that stearate was the only fatty acid found to enhance platelet aggregation in the adrenaline-ATP system. Other studies have shown that long-chain saturated fatty acids, including stearic acid, promote thrombus formation *in vitro* (Connor and Poole, 1961) and on intravenous injection (Connor *et al.*, 1963; Soloff and Wiedeman, 1963). It has been suggested that this might be dependent upon activation of the Hageman factor (Connor *et al.*, 1963). Shore and Alpers (1963) found that stearate was the most effective of the long-chain saturated fatty acids in producing platelet damage with histamine and serotonin release. Kerr *et al.* (1965) noted that platelet aggregation was greatest with a phospholipid-stearate solution, and that the change appeared to be

irreversible. The findings in experiment 6 are in general accord with these observations, and clearly incriminate stearate as an effective agent in *in vitro* platelet aggregation.

It is difficult to explain why stearate was ineffective by itself or was unable to modify nucleotide-induced platelet aggregation, but clearly enhanced platelet aggregation in the adrenaline-nucleotide system. Kerr *et al.* (1965) and Haslam (1964) were able to induce platelet aggregation by the addition of sodium stearate alone, but the concentrations of stearate used in experiment 6 were considerably weaker than those employed by these workers. Furthermore, washed platelets were used by Haslam. Until more is known of platelet nucleotide metabolism, and platelet enzyme systems, any explanations for this phenomenon must remain speculative. Whatever the mechanism, the possible biological significance of stearic acid as a factor in *in vitro* platelet aggregation and in thrombosis clearly merits exploration. In view of the non-physiological nature of the preparations used, albumin bound fatty acids are now being investigated. Nevertheless, one cannot dismiss the possibility that a dissociation from plasma proteins may occur in some circumstances *in vivo* and if so this property of stearic acid may prove to be significant. It was mentioned earlier that this fatty acid produces and also augments the so-called "platelet sticking reaction".

PART IV

IN VITRO THROMBOSIS AND PLATELET AGGREGATION IN VARIOUS CLINICAL CONDITIONS

TABLE 10

Subject categories	Age group (years)						
	11-20	21-30	31-40	41-50	51-60	61-70	71-80
Controls	3	9	12	15	10	3	-
Myocardial infarct:							
Anticoagulant	-	-	1	4	15	3	1
No anticoagulant	1	-	-	1	4	14	6
Total myocardial infarct	1	-	1	5	19	17	7

Age distribution of the 102 subjects studied. Patients with myocardial infarction are subdivided into those receiving or not receiving anticoagulant therapy.

PART IV - IN VITRO THROMBOSIS AND PLATELET AGGREGATION IN VARIOUS CLINICAL CONDITIONS

In these experiments, the Chandler apparatus has been used as an in vitro model for the study of thrombosis and spontaneous platelet aggregation in an attempt to determine if the circulating blood of patients with, after, or prone to thrombosis, shows any propensity to abnormal thrombosis or abnormal spontaneous platelet aggregation. Subjects with myocardial infarction, diabetes mellitus and postoperative patients have been studied.

Experiment 7: In vitro thrombosis and platelet aggregation in myocardial infarction

The results of this study have been reported in part in the British Medical Journal (see Appendix F).

The type of study contained in experiments 7 to 9 depends upon demonstrating differences between the mean values of the various results for the group of patients and control subjects. The same group of control subjects was used in each experiment, but will only be described in detail in this experiment.

1. Materials and Methods

Subjects studied

The age distribution of the 102 subjects studied is detailed in Table 10. Control subjects consisted of colleagues, laboratory assistants, and volunteer hospital porters in whom there was neither clinical nor electrocardiographic evidence of myocardial

infarction. Patients with myocardial infarction were selected from both hospital in-patient and out-patient clinics, and all had clinical, laboratory, and electrocardiographic evidence consistent with this diagnosis. Ten of the 50 patients with myocardial infarction and 6 of the 52 controls were women, numbers which were too small to permit a separate sex analysis. Most subjects were studied for six or seven consecutive days.

Blood samples

Antecubital venous blood was collected each morning with the aid of venous occlusion and mild forearm exercise, by means of siliconized glass syringes and 19-gauge needles. Nine ml. of blood were added to one ml. of 3.8% trisodium citrate in siliconized glass centrifuge tubes, and were mixed gently by inversion. Citrated platelet-rich plasma was prepared by centrifuging at 350 g for 15 minutes. On the first day of study, samples for total serum cholesterol and plasma fibrinogen were collected.

Total serum cholesterol was estimated with a Technicon Auto-Analyzer, a slight modification of the method described by Zlatkis, Zak and Boyle (1953) being used.

Plasma fibrinogen was determined by the method of Coles and Roman (1957).

The Chandler apparatus

This was modified from that first described by Chandler in 1958. A circular disk of perspex was attached to a larger perspex disk inclined at 30 degrees from the horizontal and mounted on an electrically driven motor rotating at 9 r.p.m. (Fig. 67).

One millilitre of citrated whole blood or platelet-rich plasma was placed in a closed circular loop of polyvinyl chloride tubing (Portex, NT/F) having an internal bore of 3 mm. This was recalcified by the addition of 0.1 ml. of M/4 calcium-chloride solution, at which time a stopwatch was started. The tube was closed with a short cuff of slightly larger tubing (Portex, NT/K), the closed loop fitted around the smaller perspex disk, and the motor started. The latter disk had a short recess to accommodate the cuff (Fig. 67). Each loop had a length of 37.9 cm. and with rotation the column of blood or plasma had a constant linear velocity of 340 cm./min. A fixed protractor was mounted beneath the perspex disk, and the angle of the advancing column of blood or plasma was recorded at 30-second intervals. Changes occurring at the advancing edge were observed through a wide-angle stereoscopic dissecting microscope under bright oblique illumination (Fig. 67).

All experiments were performed at room temperature maintained within the range 22-25°F.

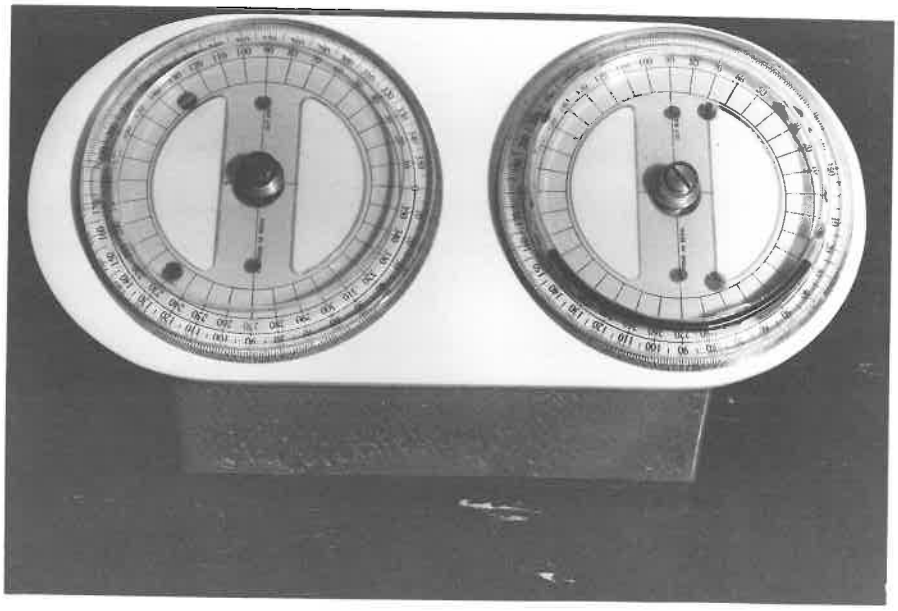


Fig. 67. The modified Chandler apparatus.

Thrombus formation time

This term, coined by Connor and Poole (1961), relates to the time at which the angle of the advancing edge of blood or plasma changed.

Whole-blood thrombus length

In all cases, rotation continued for seven minutes after an angle change had occurred. The thrombus formed was emptied into a Petri dish containing 0.9% saline, and its length was measured to the nearest millimetre. Mean thrombus length for each patient was the mean of the values obtained on each of the days studied.

Whole-blood thrombus weight

After fixation in 10% formalin the thrombi were blotted dry between filter papers and weighed to the nearest milligram. Mean thrombus weight for each patient was again the mean of the values obtained on each of the days studied.

Platelet aggregation

Platelet-rich plasma was observed in the transparent plastic tube through a dissecting microscope at 12 magnifications. The time at which visible platelet aggregates formed, and their number, size and time of persistence, were recorded. Three patterns of spontaneous platelet aggregation were recognized:

(a) Normal pattern was defined as a sporadic appearance and disappearance of visible aggregates rarely exceeding

one or two in number and persisting only for one minute or less before a change in the angle of the advancing column of plasma occurred. Just before this angle change there was invariably a rapid and dramatic rise in the number of aggregates, which increased in size and coalesced to form a solid mass at the advancing edge.

(b) Persistence was defined as the continuous presence of visible platelet aggregates for a minimum of four minutes. In many cases aggregates formed within one or two minutes of recalcification and persisted throughout the experiment.

(c) "Snowstorm" phenomenon was defined as the formation of hundreds or thousands of small platelet aggregates often within one to two minutes of recalcification. These invariably persisted for at least four minutes. On a number of occasions the process was either completely or partially reversible. This phenomenon should not be confused with the large number of aggregates seen normally just prior to a change in the angle of the advancing column of plasma.

Statistical methods employed in part IV

A linear regression to determine age dependence was made for each of the variables measured. Significant results (at the 5% level) were obtained only for fibrinogen, mean thrombus length and mean thrombus weight. For each of these factors, tests were made

TABLE 11

Showing regression lines for fibrinogen, mean thrombus length and mean thrombus weight, where test of significance of regression coefficient yielded a significant value.

DEPENDENT VARIABLE	SUBJECT CATEGORY	REGRESSION LINE*
Fibrinogen	Total diabetes mellitus	log. Y = 5.875 + 0.0087 (X-58.60)
	Total uncomplicated diabetics	log. Y = 5.719 + 0.0120 (X-57.03)
	Uncomplicated diabetics, insulin	log. Y = 5.617 + 0.0110 (X-50.33)
Mean Thrombus Length	Control subjects	log. Y = 2.955 = 0.0062 (X-41.54)
	Total myocardial infarct	log. Y = 3.584 + 0.0119 (X-59.52)
	Myocardial infarct, no anticoagulant	log. Y = 3.626 + 0.0133 (X-63.35)
	Total diabetes mellitus	log. Y = 3.683 + 0.0062 (X-58.81)
	Total uncomplicated diabetics	log. Y = 3.477 + 0.0081 (X-57.15)
	Uncomplicated diabetics, insulin	log. Y = 3.488 + 0.0090 (X-50.32)
	Total post operative	log. Y = 3.938 + 0.0074 (X-54.92)
	Post operative, score $\leqslant 3$	log. Y = 3.842 + 0.0075 (X-49.97)
Mean Thrombus Weight	Control subjects	log. Y = 4.251 + 0.0067 (X-41.54)
	Total myocardial infarct	log. Y = 4.816 + 0.0097 (X-59.52)
	Total diabetes mellitus	log. Y = 4.769 + 0.0083 (X-58.81)
	Total uncomplicated diabetics	log. Y = 4.561 + 0.0097 (X-57.15)
	Uncomplicated diabetics, insulin	log. Y = 4.577 + 0.0114 (X-50.32)
	Complicated, non-vascular, diabetics	log. Y = 5.192 + 0.0144 (X-53.50)
	Total post operative	log. Y = 5.085 + 0.0066 (X-54.92)
	Post operative, score $\leqslant 3$	log. Y = 4.995 + 0.0062 (X-49.97)

(continued)

TABLE 11 (continued)

* Regression lines of form: log. $Y = a + b(X-\bar{X})$
where Y = value of dependent variable i.e. fibrinogen, mean thrombus length or mean thrombus weight

X = age

a = mean log. Y value

b = regression coefficient

$\bar{X}$ = mean X value

TABLE 12

Mean values (age corrected where necessary) for fibrinogen, mean thrombus length and mean thrombus weight used in tests of equality of means in the various subject categories indicated.

SUBJECT CATEGORY	FIBRINOGEN			MEAN THROMBUS LENGTH			MEAN THROMBUS WEIGHT		
	Mean Value*	% Increase**	Significance	Mean Value	% Increase	Significance	Mean Value	% Increase	Significance
Controls	233.7	76	P<0.001	20.71	63	P<0.001	75.23	53	P<0.001
Myocardial Infarct (MI)	412.0			33.27			114.8		
MI on anti-coagulant	425.5	7	N.S.#	36.11	0.7	N.S.	117.7	9.5	N.S.
MI off anti-coagulant	396.7			35.86			128.9		
Controls	252.4	34	P<0.001	20.48	86	P<0.001	76.29	47	P<0.001
Diabetics	339.4			38.15			111.9		
Uncomplicated Diabetics	308.6	31	—##	32.64	45	P<0.001	96.79	45	P<0.001
Complicated Diabetics	405.5			47.40			140.2		
Complic. Vasc. Diabetics	390.8	15	N.S.	42.46	44	P<0.01	123.6	54	P<0.01
Complic. Non-Vasc. Diab.	449.5			61.35			190.2		

(continued)

TABLE 12 (continued)

SUBJECT CATEGORY	MEAN THROMBUS LENGTH			MEAN THROMBUS LENGTH			MEAN THROMBUS LENGTH		
	Mean Value*	% Increase**	Signifi-cance	Mean Value	% Increase	Signifi-cance	Mean Value	% Increase	Signifi-cance
Controls	233.7	96	P<0.001	20.22	143	P<0.001	73.64	111	P<0.001
Post operative	458.2			49.13			155.3		
Post operative Score ≤ 3	405.6	34	P<0.01	47.97	17	N.S.	151.8	16	N.S.
Post operative Score > 3	542.5			55.90			176.0		

*The mean values shown are antilogarithms of mean log. Y values, where Y is the dependent variable (fibrinogen, mean thrombus length or mean thrombus weight), age corrected where necessary.

** % increase $= \frac{\text{difference in mean values}}{\text{lesser mean value}} \cdot 100$

N.S. = not significant

No comparison possible

of equality of regression coefficients for the groups, and, when these were found to show no significant differences, analyses of covariance were performed, to test equality of age corrected means. A log transformation was applied to the original data to remove skewness throughout these tests. The methods employed in tests of equality of means for fibrinogen, mean thrombus length and mean thrombus weight in the various categories of subjects studied, are summarised in Appendix H.

Where the regression was non-significant for all groups, a square root or log transformation was used, when necessary, to remove skewness before Student's "t" or Behren's tests were applied to test equality of means.

Where a test of significance of regression coefficient yielded a significant value, the regression lines have been shown (Table 11). In all other cases, the mean values shown in Table 12 were used in tests of equality.

Correlations between different variables were determined by the product moment correlation coefficient.

I am indebted to Miss M. Butcher for invaluable assistance in the statistical analyses incorporated in Tables 11, 12 and 15, to Professor A. T. James for his advice on this aspect, and to Mrs. J. Parfitt for some of the data analysis involved.

2. Results

Whole-blood thrombus length and weight

Figure 68 shows the mean thrombus length (mm.) and mean thrombus weight (mg.) in control subjects and patients with myocardial infarction. Patients with myocardial infarction are subdivided into those receiving or not receiving anticoagulant therapy.

Thrombus length ($P < 0.001$) and weight ($P < 0.001$) were both significantly greater in patients with myocardial infarction than in control subjects, but there were no significant differences in thrombus length and weight in patients receiving anticoagulant therapy, and in patients not receiving anticoagulant therapy.

Effect of time after infarction

Figure 69 shows the mean thrombus length (mm.) and mean thrombus weight (mg.) in patients with myocardial infarction analysed according to the time after infarction and the presence or absence of anticoagulant therapy. Thrombus length and weight clearly declined with the passage of time after infarction, irrespective of the presence or absence of anticoagulant therapy. In patients six or more weeks after the clinical event the values for thrombus length and weight had returned to within the normal range in only 7 of the 12 subjects studied.

Plasma fibrinogen and thrombus size

Plasma fibrinogen levels were found to be significantly elevated ($P < 0.001$) in patients with myocardial infarction (Fig. 70). Both in

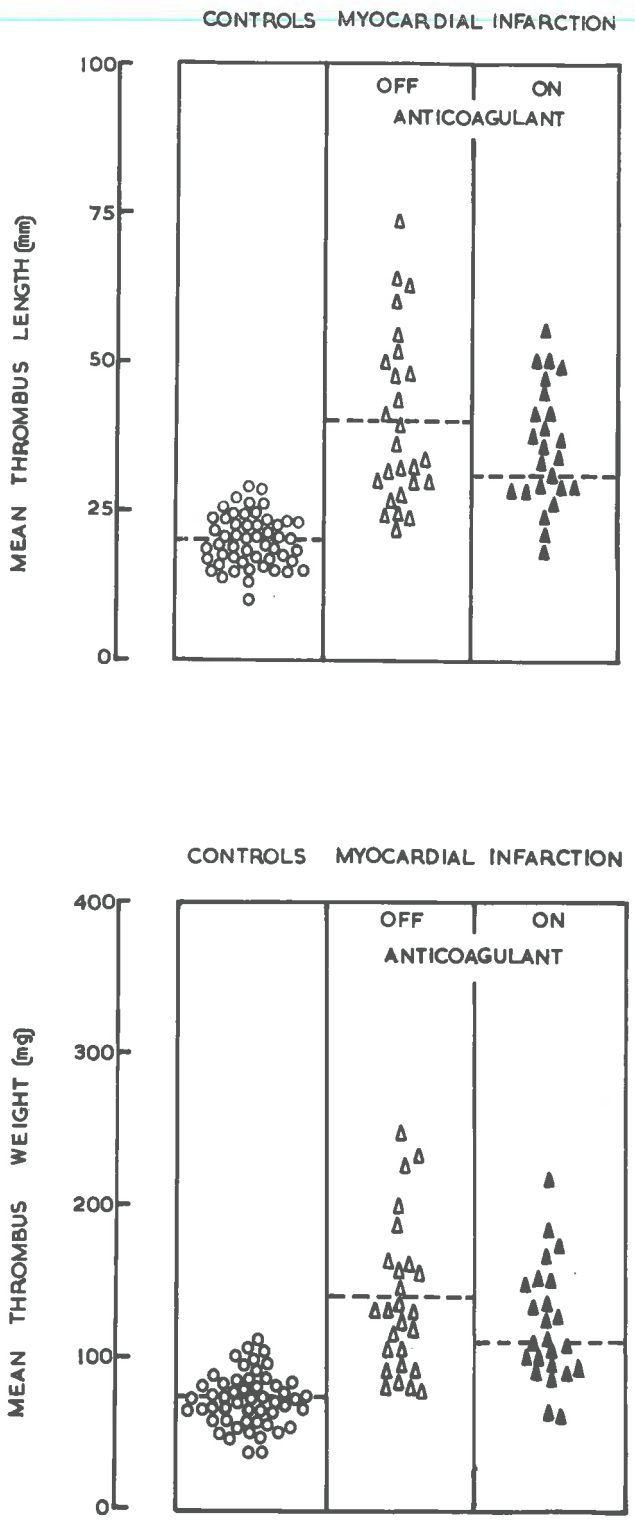


Fig. 68. Mean thrombus length (TOP) and weight (BOTTOM) in control subjects and in patients with myocardial infarction subdivided into those receiving and not receiving anticoagulant therapy.

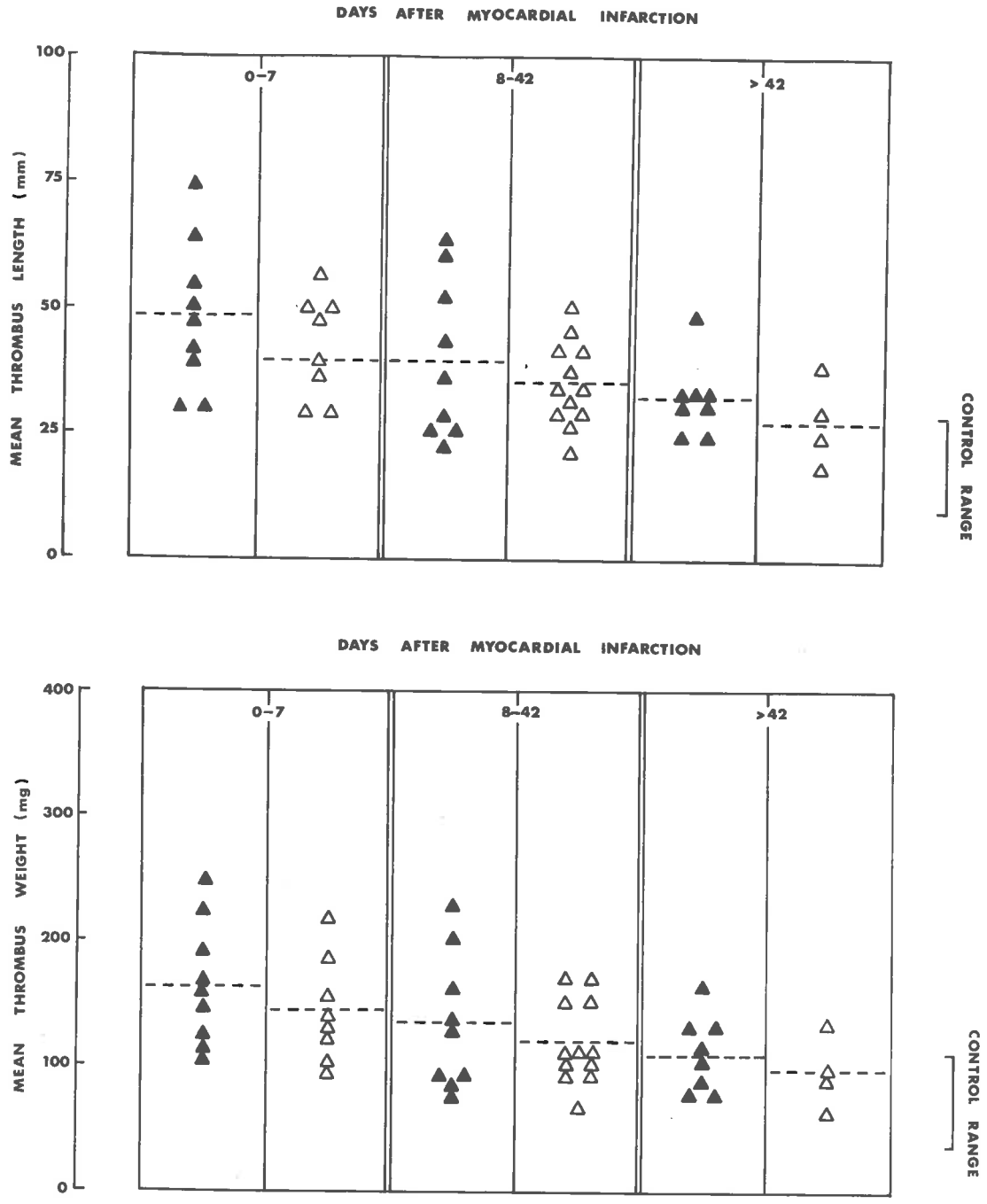


Fig. 69. The mean thrombus length (TOP) and weight (BOTTOM) in patients with myocardial infarction are analysed according to time after infarction and the presence (△) or absence (▲) of anticoagulant therapy. The range of values for thrombus length and weight in control subjects is indicated on the right of each figure.

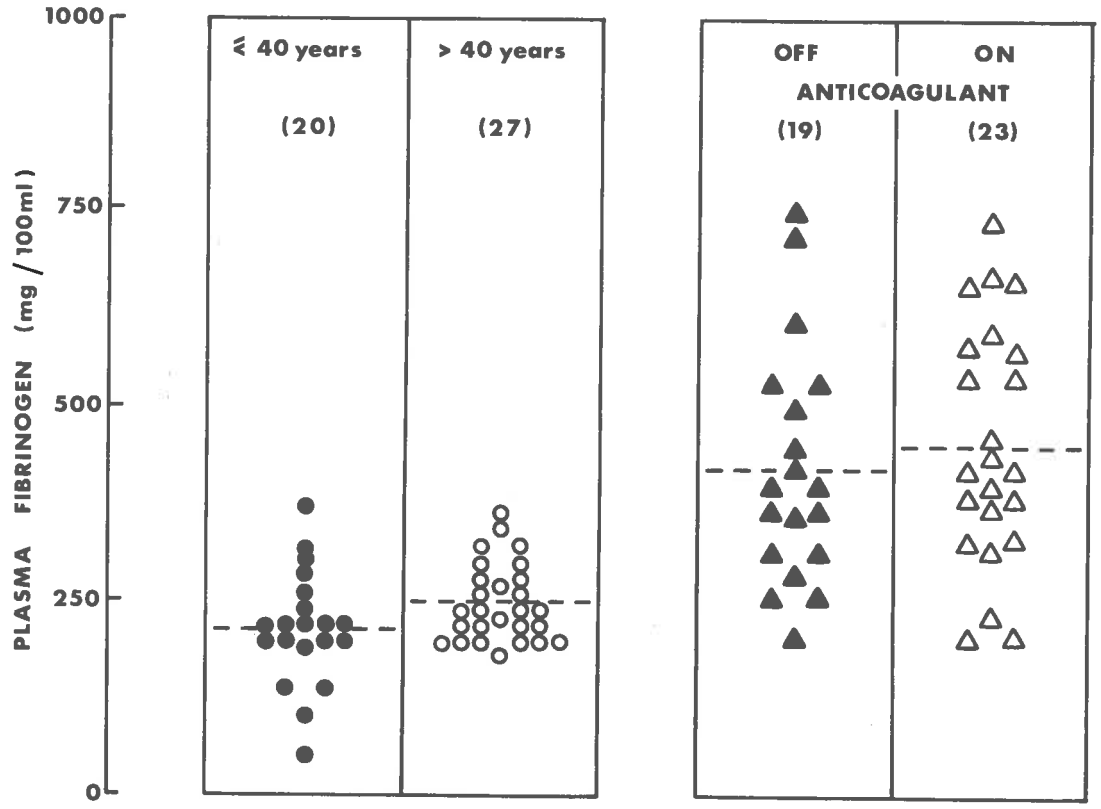


Fig. 70. Plasma fibrinogen level (mg/100 ml) in control subjects aged 40 or less and over 40, and in patients with myocardial infarction subdivided into those receiving and not receiving anticoagulant therapy. The number of subjects in each group is indicated at the top of each column.

these patients and in control subjects there was a statistically significant correlation between the plasma fibrinogen level on the one hand and thrombus weight on the other (Fig. 71). In the case of patients with myocardial infarction the calculated correlation coefficient r was 0.7116 ($P < 0.001$), and in the controls r was 0.4580 ($P < 0.01$).

Total serum cholesterol and thrombus size

Neither in patients with myocardial infarction nor in healthy controls was there a significant correlation between thrombus weight (or length) and the total serum cholesterol level (Fig. 72). No significant difference was noted between total serum cholesterol levels in patients with myocardial infarction and control subjects.

Thrombus formation time

In patients with myocardial infarction both the whole blood ($P < 0.01$) and plasma ($P < 0.001$) thrombus formation times were significantly longer than in control subjects (Fig. 73). There was no significant difference in the thrombus formation times in patients with myocardial infarction receiving oral anticoagulant therapy compared with patients not receiving anticoagulant therapy, a finding which is not in agreement with the results of Cunningham, McNicol and Douglas (1965). The latter group, however, compared subjects with myocardial infarction on anticoagulant therapy, and a mixed group of control subjects including healthy colleagues.

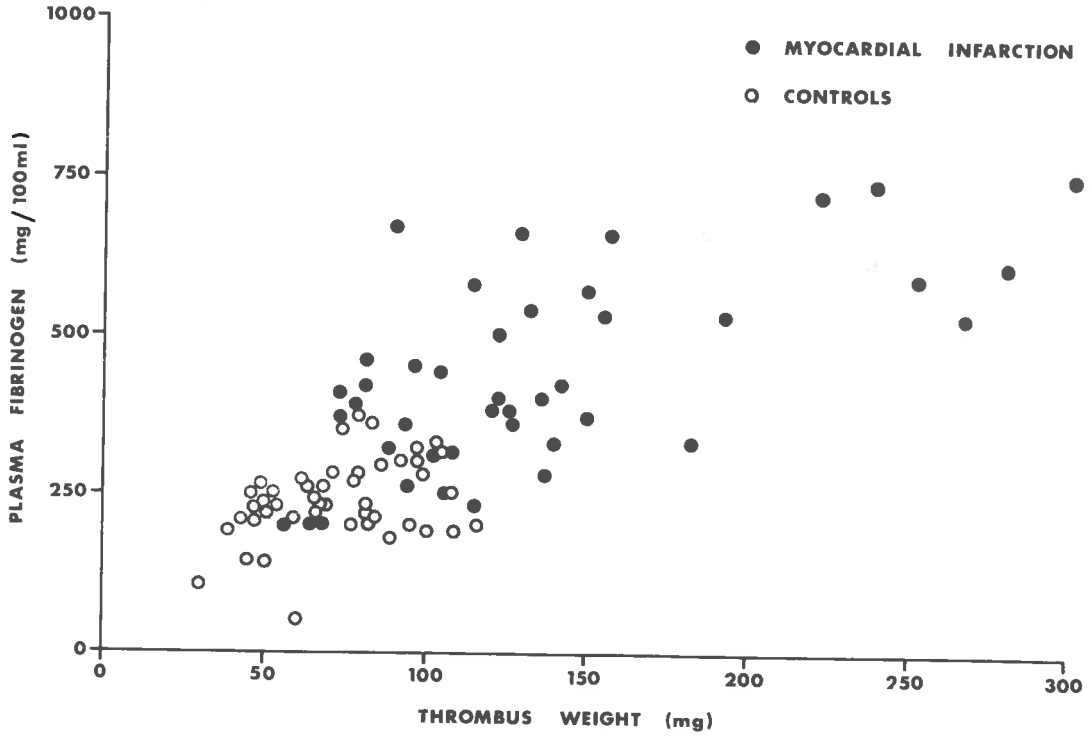


Fig. 71. Mean thrombus weight (mg) plotted against plasma fibrinogen level (mg/100 ml) in patients with myocardial infarction (●) and in control subjects (○).

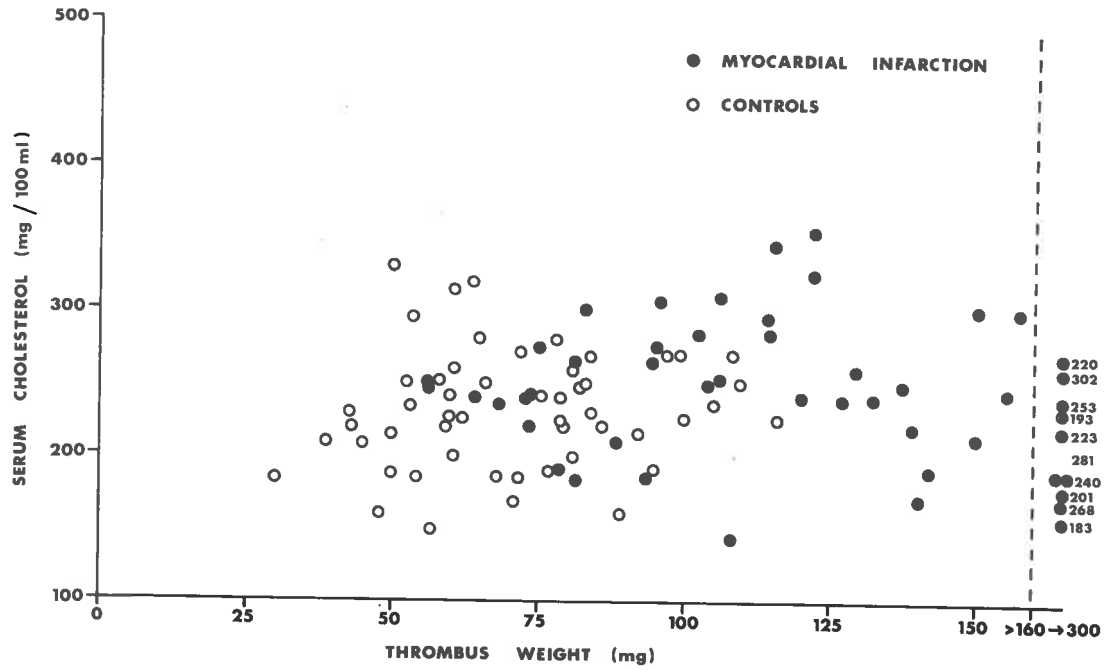


Fig. 72. Mean thrombus weight (mg) plotted against total serum cholesterol (mg/100 ml) in patients with myocardial infarction (●) and in control subjects (○).

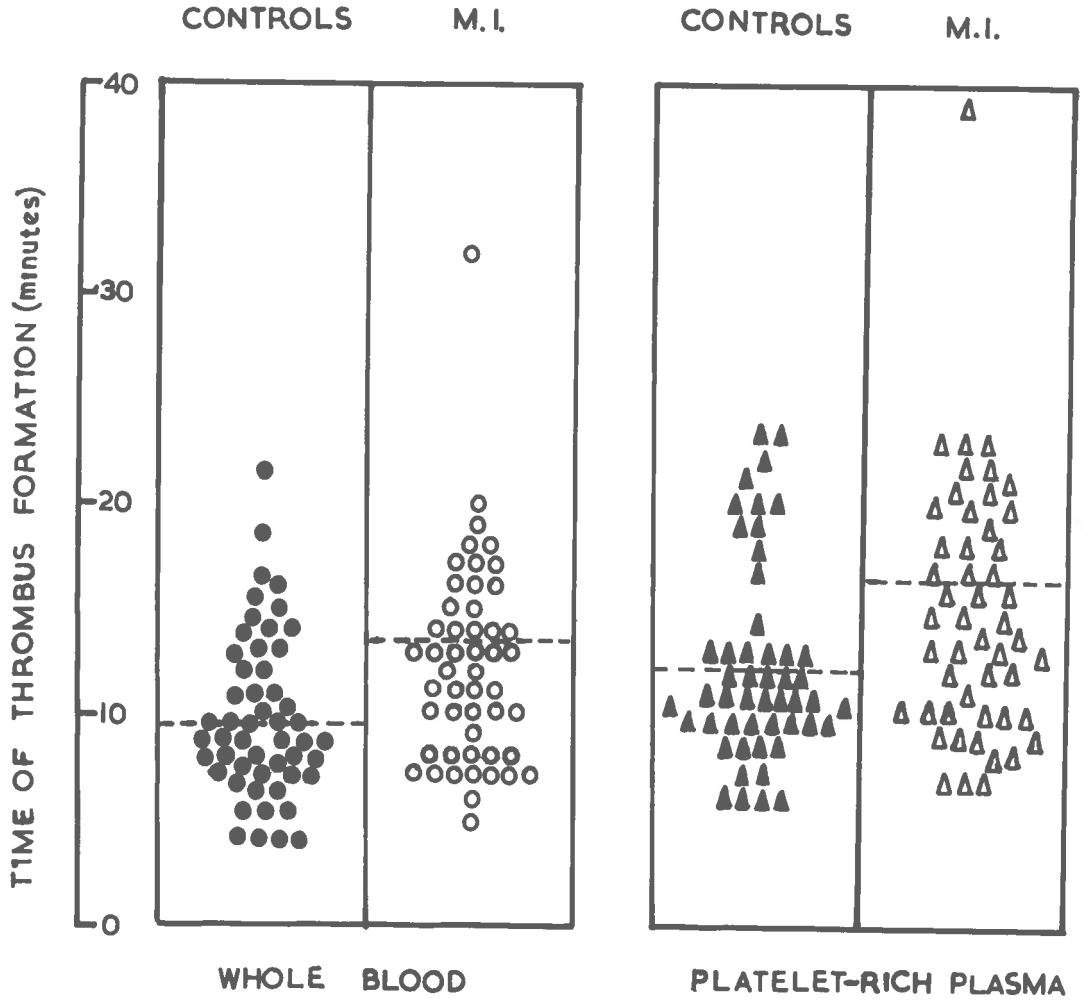


Fig. 73. Whole blood and plasma thrombus formation times in control subjects and in patients with myocardial infarction.

TABLE 13

Subject categories	Days studied	Persistence (%)	Snowstorm phenomenon (%)	Total platelet abnormality (%)
Controls	303	3.0	1.3	4.3
Myocardial infarct:				
On anticoagulant	148	16.2	11.5	27.7
No anticoagulant	169	20.7	4.7	25.4
Myocardial infarct combined	317	18.6	7.9	26.5

Frequency (expressed as a percentage of the number of days studied) of the two patterns of abnormal platelet aggregation considered separately and together, in control subjects, and in patients with myocardial infarction subdivided into those receiving and not receiving anticoagulant therapy, and combined.

Neither in patients with myocardial infarction nor in healthy controls was there a significant correlation between whole blood or plasma thrombus formation times and the plasma fibrinogen or total serum cholesterol level.

Platelet-rich plasma

In control subjects the normal pattern (see Methods) of platelet aggregation was observed on 95.7% of days studied (Table 13). Persistence of platelet aggregates was noted on 3.0% of 303 days studied, and the snowstorm phenomenon occurred on 1.3% of days studied, giving a total of 4.3% of days showing abnormal platelet aggregation.

In contrast to the findings in control subjects, abnormal platelet aggregation was observed in patients with myocardial infarction with an overall frequency of 26.5% of 317 days studied (Table 13). Persistence accounted for 18.6%, and the snowstorm phenomenon for 7.9% of the days studied showing abnormal platelet aggregation. Table 13 also shows the percentage prevalence of days of abnormality in patients with and without anticoagulant therapy. The overall frequency of abnormal platelet aggregation was found to be 27.7% and 25.4% in patients on and off anticoagulants respectively. Persistence occurred less often in patients receiving anticoagulants (16.2%) than in those not receiving anticoagulants (20.7%), but the snowstorm phenomenon was more frequent in patients on anticoagulants (11.5%)

TABLE 14

Myocardial infarct category	Time after infarction (days)											
	0-7				8-42				> 42			
	Days studied	Persistence (%)	Snow-storm phenomenon (%)	Total platelet abnormality (%)	Days studied	Persistence (%)	Snow-storm phenomenon (%)	Total platelet abnormality (%)	Days studied	Persistence (%)	Snow-storm phenomenon (%)	Total platelet abnormality (%)
On anticoagulant	47	21.3	14.9	36.2	76	18.4	13.2	31.6	25*	0.0*	0.0*	0.0*
No anticoagulant	52	21.2	1.9	23.1	51	25.5	3.9	27.5	66	18.2	7.6	25.8
Combined	99	21.2	8.1	29.3	127	20.5	9.4	29.9	91	13.2	5.5	18.7

*Only four patients in this group

Frequency (expressed as a percentage of the number of days studied) of the two patterns of abnormal platelet aggregation (considered separately and together), according to time after the event, in patients with myocardial infarction, the latter being subdivided into those receiving and not receiving anticoagulant therapy, and combined.

than in those not receiving anticoagulants (4. 7%).

When the frequency of abnormal platelet aggregation was considered according to the number of subjects manifesting either pattern of abnormal aggregation on one or more days of study, obvious differences still emerged between control subjects and subjects with myocardial infarction. Abnormal platelet aggregation occurred in 15. 4% of control subjects, whereas 76. 0% of patients with myocardial infarction showed this phenomenon. (There were similar differences between control subjects and the diabetic and postoperative categories, and these results have all been summarised in Appendix J.)

The frequency of abnormal platelet aggregation has also been considered in relation to the time after infarction (Table 14). It can be seen that the overall frequency of platelet abnormality was practically the same at 0-7 days (29. 3%) and 8-42 days (29. 9%), but in patients six or more weeks after infarction the number of days showing abnormality had decreased to 18. 7%. When the two patterns of abnormal platelet aggregation are considered separately (Table 14) it is apparent that the frequency of persistence 0-7 days after infarction (21. 2%) was similar to the frequency at 8-42 days (20. 5%) after infarction, but in patients who had sustained infarcts more than six weeks before the time of study only 13. 2% of days studied showed this pattern. The frequency of the snowstorm phenomenon varied from 8. 1% at 0-7 days to 9. 4% at 8-42 days, while in patients with infarcts over six weeks old the number

of days studied showing this phenomenon decreased to 5.5%. It is of interest to note that neither persistence nor the snowstorm phenomenon was observed in anticoagulated patients six or more weeks after infarction. However, in this group only four subjects were studied for a total of 25 days, and therefore no definite conclusions can be drawn from this observation.

There was no correlation between abnormal platelet behaviour and the plasma fibrinogen level in either control subjects or patients with myocardial infarction.

DISCUSSION

In his original description Chandler (1958) found that the whole column of blood eventually became static and moved around in the direction of rotation of the tube. In 1959 Poole slightly modified this apparatus and found, instead, that a small solid object formed just behind the advancing edge of the column of blood, and the whole column never solidified completely. In Chandler's original method the turn-table was inclined at 23° to the horizontal while in Poole's modified technique it rotated in a near vertical plane. Assuming that the differences could be due to the variation in the inclination of the turn-table, the rotating disc in this experiment was inclined at 30°.

The most important factor, however, in the design of the experiment, proved to be the length of time, and the speed at which the

blood was centrifuged in preparing the sample of platelet-rich plasma. A series of experiments were conducted to determine the effect of varying platelet counts on the pattern of spontaneous platelet aggregation. After centrifuging at 350 g. for 3 minutes, platelet-rich plasma from all subjects, be they patients or controls, showed a massive snowstorm phenomenon with fusion of platelet aggregates to form a white mass of considerable proportion several minutes before the time of thrombus formation. If spun for 6 minutes the same phenomena were observed in patients, but in control subjects the snowstorm phenomenon was now reversible. After 9 minutes of centrifugation there might be a reversible snowstorm phenomenon in the patient, but the response was invariably normal in all control subjects studied. To clearly distinguish between patients with in vitro abnormalities and control subjects the time of 15 minutes for centrifugation was selected. This time is well in excess of 9 minutes which proved to be satisfactory in distinguishing normal from abnormal patterns and it is therefore quite likely that the incidence of abnormal spontaneous platelet aggregation in patients with myocardial infarction would have been significantly greater had a shorter spinning time been used. It must be emphasized that in these experiments the patients and controls chosen were those respectively manifesting in vitro abnormalities and no abnormalities after 15 minutes of centrifugation, in order that the above comparisons could be made at varying times

after centrifugation.

Hirsh, McBride and Dacie (1966) suggested that the elevated platelet count after splenectomy results in an increase in the total adhesive platelet count. This concept would account for the above observations. In any blood sample spun for only 3 minutes, the platelet count is so high and consequently the number of "adhesive" or sticky platelets so abundant that a snowstorm phenomenon develops. However when spun for 15 minutes the platelet count, although variable is less than 350, 000 per cu. mm. and quite often less than 200, 000 per cu. mm. When the count is less than 350, 000 per cu. mm. in apparently healthy control subjects the number of sticky platelets is so low that abnormal spontaneous platelet aggregation is not observed. However with platelet counts of less than 200, 000 per cu. mm. a snowstorm phenomenon was observed in some of the subjects with myocardial infarction, indicating that the percentage of adhesive platelets must be higher in these subjects.

It has been demonstrated that the thrombi produced in the Chandler apparatus with blood from patients after myocardial infarction are both longer and heavier than the thrombi of control subjects having neither clinical nor electrocardiographic evidence of infarction. Thrombus size clearly decreased with the passage of time after infarction. There was no significant difference in thrombus size in those patients receiving anticoagulant therapy, and those patients not

receiving therapy. Both in the control subjects ($P < 0.01$) and in patients with myocardial infarction ($P < 0.001$) thrombus weight and the plasma fibrinogen levels showed statistically significant correlations. It is well known that the plasma fibrinogen level is elevated in patients after myocardial infarction (Meyers, 1948), a finding which has been confirmed in this study. This elevation could either wholly or partly account for the longer and heavier thrombi found in patients with myocardial infarction.

It cannot be determined whether the factor or factors responsible for the development of large thrombi were present before infarction or merely reflect the results of infarction. It appears that the latter is more likely. An increase in plasma fibrinogen occurs in association with tissue destruction or inflammation (Ham and Curtis, 1938). It seems likely that the elevated fibrinogen level and therefore the increase in thrombus size could both be sequelae of the myocardial infarct. However, thrombus size increases with age in apparently healthy subjects, and this observation suggests that the factor or factors responsible for this phenomena are present to some extent before the clinical event, and that coronary thrombosis could result from an exaggeration of this trend. It is interesting to speculate whether this propensity to abnormal <u>in vitro</u> thrombosis after myocardial infarction might be associated with the tendency of these patients to subsequent thrombotic episodes.

The results clearly indicate that there is a significant increase

in the incidence of platelet abnormalities in patients with myocardial infarction when compared with control subjects. Again it is uncertain whether these findings relate to a state existing before infarction or are wholly or in part a sequel to myocardial necrosis.

The overall frequency of platelet abnormality was similar in patients with myocardial infarction whether or not they were receiving anticoagulant therapy (Table 13). Two explanations for this finding should be considered. First, it could be that in anticoagulated patients the initial overall frequency of platelet abnormality was greater, and that this frequency has in fact been reduced by therapy. Alternatively, anticoagulants may have had little or no effect on platelet aggregation. On the other hand, there was a trend (Table 13) for the snowstorm phenomenon to occur more often in anticoagulated patients (11.5%) than in those not receiving anticoagulant therapy (4.7%). At first sight this might suggest that anticoagulant therapy had enhanced platelet aggregation. This trend may, however, merely reflect the fact that those patients selected for anticoagulant therapy differed from those not given anticoagulants, possibly on the basis of more extensive infarction. It is apparent that the effect of oral anticoagulants on platelet aggregation in this <u>in vitro</u> system is inconclusive.

Experiment 8: Platelet behaviour and in vitro thrombosis in diabetes mellitus

The subject of diabetes mellitus and vascular disease was reviewed in part I. It would appear that both clinical and preclinical diabetes mellitus are in some way associated with the development of occlusive arterial disease. This relationship could reflect an enhanced atheregenesis in diabetic subjects, an increased thrombotic tendency or both. In this experiment the possibility of an increased thrombotic tendency is explored, using the Chandler apparatus. In addition some preliminary observations on the effects in healthy subjects of intravenous glucose on both platelet count and platelet behaviour are reported.

1. Material and Methods

The Chandler apparatus and the technique employed, and the estimation of total serum cholesterol and plasma fibrinogen level have been detailed in the previous experiment.

Blood sugar

The blood sugar levels were estimated in a Technicon Auto Analyser using the ferricyanide method (Hoffman, 1937).

Platelet counts

Platelets were counted using phase contrast microscopy (Brecher and Cronkite, 1950).

Glucose infusion studies using radioactive platelets

Three hundred millilitres of blood were collected into a double plastic pack (Tuta Products Ltd., Sydney, Australia) containing an appropriate amount of acid-citrate-dextrose anticoagulant (Aster and Jandl, 1964). Platelets were isolated from whole blood by differential centrifugation at 4°C and labelled with radioactive disodium chromate (Cr^{51}), using a technique which has been described in detail previously (Davey and Lander, 1963). The cells were resuspended in plasma to a final volume of 22 to 25 ml. A known volume (20 ml.) of the suspension was then injected intravenously into an antecubital vein from a siliconized syringe. Twenty four hours later when platelet radioactivity had reached equilibrium in the circulation a glucose infusion study was carried out with the patient lying supine. Scintillation detectors were placed over the liver and spleen (6 patients); liver, lung and spleen (1 patient) and spleen and lung (1 patient). A continuous surface scan was performed for the duration of the study to detect possible alterations in platelet radioactivity in the various organs. Preinfusion samples of blood were collected for estimations of blood sugar, whole blood platelet count and platelet radioactivity.

Fifty millilitres of 50% glucose solution were infused over a period of approximately 3 minutes and further samples of blood were taken at 5, 20 and 45 minutes after completion of the infusion. Estimations of blood glucose, whole blood platelet count and platelet

radioactivity were determined on each specimen.

Preparation of samples and estimation of platelet radioactivity

Platelets were isolated from samples of whole blood using a silicone flotation technique (Morgan and Szafir, 1961). Platelet radioactivity was measured with a precision of $\pm$ 1% by scintillation detection in a well-type crystal adapted to a Philips Universal gamma spectrometer. Specimens were counted at the conclusion of each study to obviate the necessity for applying decay corrections.

Subjects studied

The age-and-sex distribution of the 83 diabetic and 52 healthy control subjects is detailed in Figure 74. The same healthy control subjects were used as in experiment 7. The diabetic subjects were selected at random from hospital in- and out-patient clinics, and in all of these a definite diagnosis of diabetes mellitus had been established.

Because of the episodic nature of the platelet abnormalities noted in patients with myocardial infarction (experiment 7) an attempt was made to study each subject for 6-7 consecutive days. Those studied for less were discharged from hospital before completion of the study. Thus the 52 healthy controls were studied for a total of 303 days, and the 83 diabetic patients for a total of 354 days. It can be seen (Fig. 74) that the control and diabetic groups are incompletely matched for age and sex, due largely to the difficulty of obtaining healthy controls over the age of 60 years.

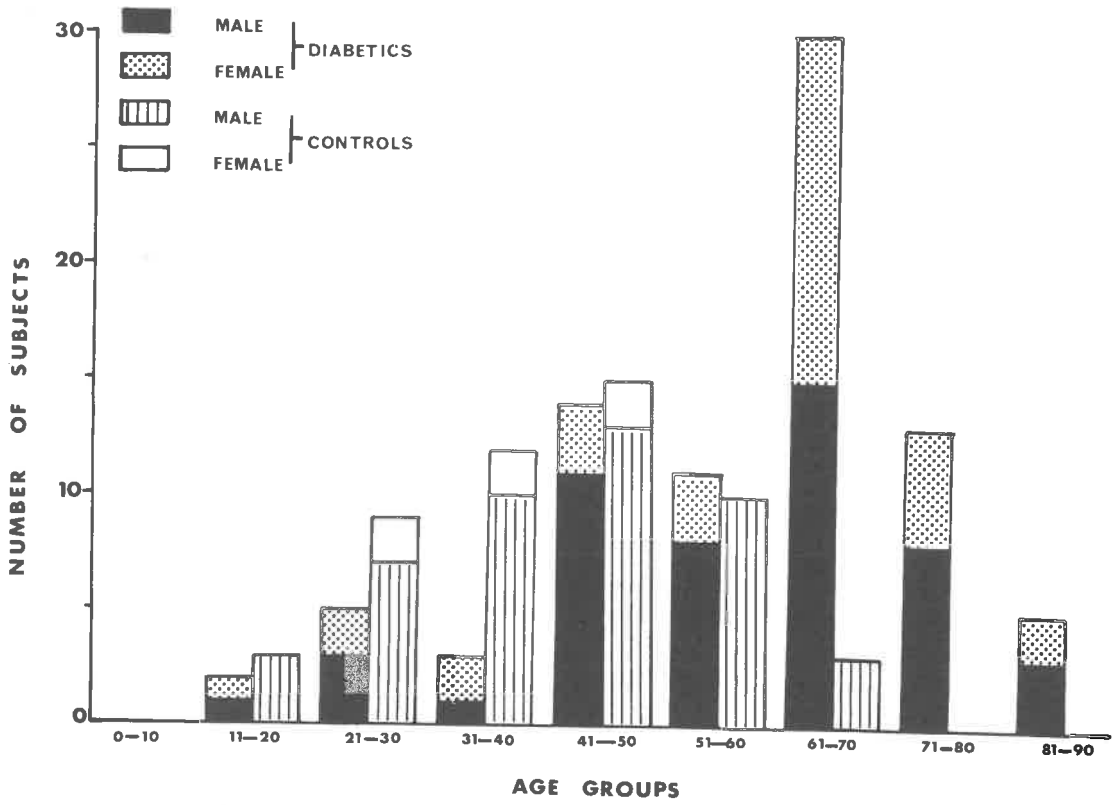


Fig. 74. Age-and-sex distribution of the control and diabetic subjects studied. The diabetes was of juvenile onset in only two instances.

The diabetics have been divided into uncomplicated and complicated groups. The latter group has been further subdivided into 2 categories, namely those with overt vascular disease, such as myocardial infarction or peripheral vascular disease, and those without overt occlusive vascular disease. Included in this subgroup are patients with diabetic retinitis, renal and peripheral nerve complications, and a large number with infections. This subgroup has been arbitrarily named "non-vascular".

2. Results

Whole blood thrombus length and weight

Figure 75 shows the mean thrombus length (mm.) and the mean thrombus weight (mg.) in control subjects and in patients with diabetes mellitus. Thrombus length and weight were significantly greater in patients with diabetes mellitus, than in controls ($P < 0.001$). Furthermore, thrombus length and weight were both significantly greater in the complicated group than in those whose diabetes was uncomplicated ($P < 0.001$). Finally, thrombus length and weight were greater in the complicated "non-vascular" than in complicated vascular diabetics ($P < 0.01$).

Thrombus size and severity of diabetes

Using the type of treatment as an arbitrary index of the severity of the diabetes, the mean thrombus length and weight in patients

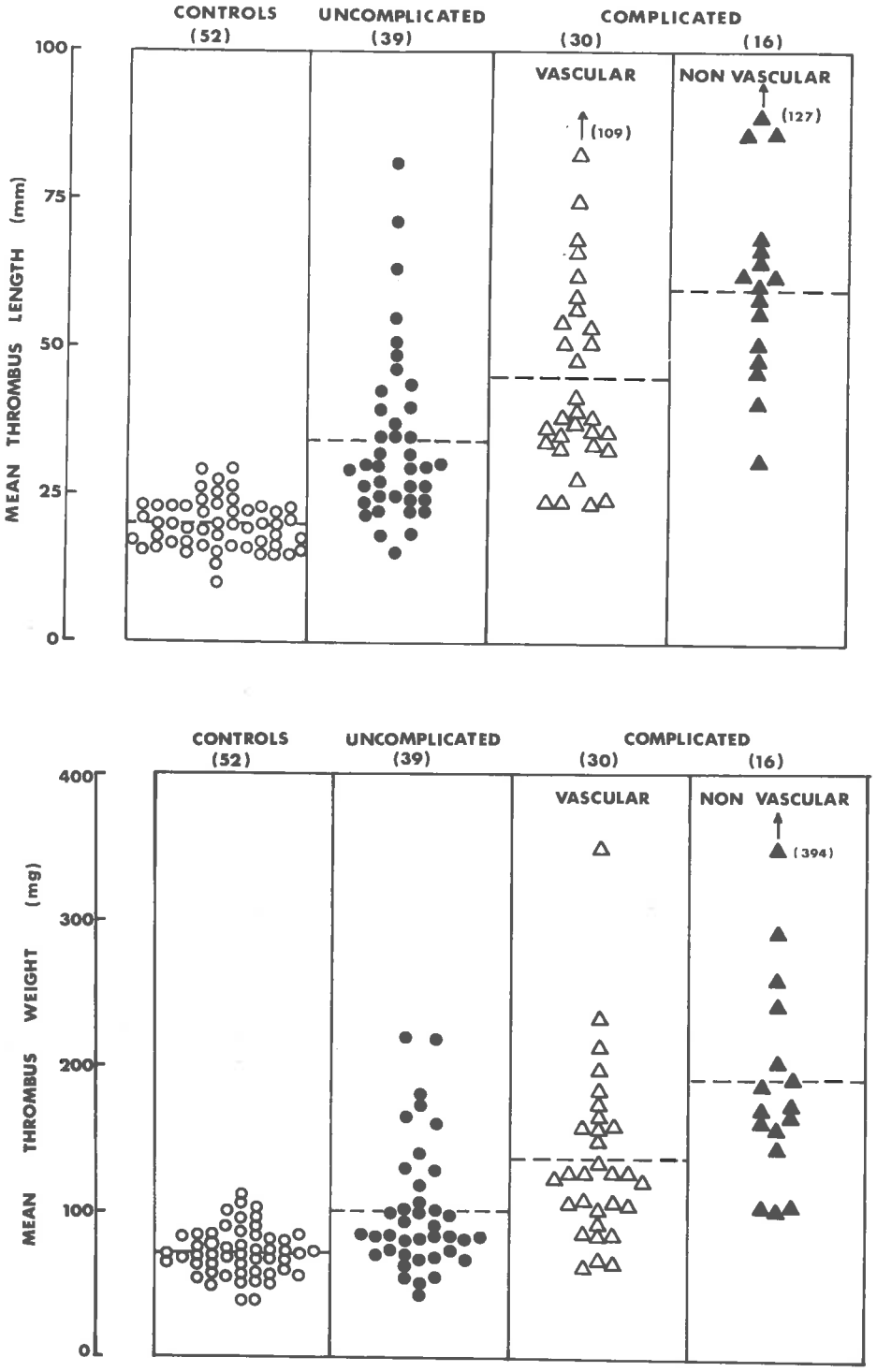


Fig. 75. Mean thrombus length (TOP) and weight (BOTTOM) in control subjects and in patients with diabetes mellitus. The figures in parentheses above each column indicate the number of subjects in each category. Although the mean thrombus length has been plotted for 85 diabetic subjects, only 83 have been considered in the text because sufficient clinical information was not available in two of the subjects.

TABLE 15

Mean values (age corrected where necessary) for fibrinogen, mean thrombus length and mean thrombus weight used in tests of equality of means in the diabetic categories indicated.

SUBJECT CATEGORY	FIBRINOGEN			MEAN THROMBUS LENGTH			MEAN THROMBUS WEIGHT		
	Mean Value*	% Increase**	Significance	Mean Value	% Increase	Significance	Mean Value	% Increase	Significance
Uncomplicated diabetes, diet	315.5	6	N.S.*	29.12	20	N.S.	83.98	26	N.S.
Uncomplicated diabetes, insulin	296.8			35.08			105.7		
Complicated diabetes, diet	393.9	8	N.S.	44.95	12	N.S.	134.4	10	N.S.
Complicated diabetes, insulin	424.2			50.18			148.3		

(continued)

TABLE 15 (continued)

*The mean values shown are antilogarithms of mean log Y values, where Y is the dependent variable (fibrinogen, mean thrombus length or mean thrombus weight), age corrected where necessary.

** % increase = $\frac{\text{difference in mean values}}{\text{lesser mean value}}$ 100

N.S. = not significant

receiving diet and oral hypoglycaemic agents on the one hand, and insulin therapy on the other were compared. Within both the complicated and uncomplicated groups the type of treatment was not associated with any significant difference in thrombus size (Table 15).

Plasma fibrinogen

Plasma fibrinogen levels were found to be elevated in patients with diabetes mellitus (Fig. 76), the difference between healthy controls and diabetics being highly significant ($P < 0.001$). There was no significant difference between the fibrinogen levels of the complicated "non-vascular" diabetics compared with those with vascular complications. Using the type of treatment as an index of severity, no significant differences in plasma fibrinogen levels were observed within the complicated and uncomplicated groups of diabetics (Table 15).

Plasma fibrinogen and thrombus size

Both in control and also in diabetic subjects, considered as a whole and in the various categories, there was a statistically significant correlation between the plasma fibrinogen level and thrombus weight. In the controls the calculated correlation coefficient r was 0.4952 ($P < 0.001$) and in patients with diabetes r was 0.7515 ($P < 0.001$); in uncomplicated diabetes $r = 0.6186$ ($P < 0.001$); in the complicated vascular $r = 0.8105$ ($P < 0.001$), and in the complicated non-vascular $r = 0.6730$ ($P < 0.001$). Similar correlations emerged for thrombus length and plasma fibrinogen levels.

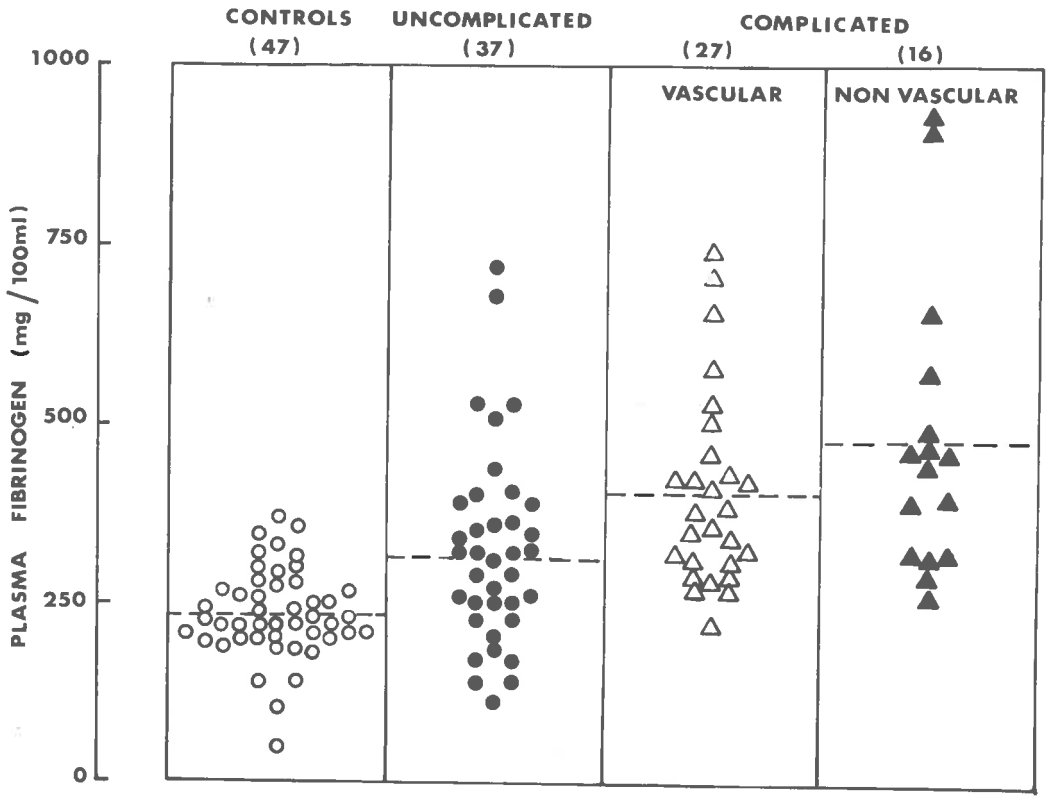


Fig. 76. Plasma fibrinogen levels in control subjects and in patients with diabetes mellitus. The diabetic categories are as described in the text.

Total serum cholesterol and thrombus size

The total serum cholesterol level was marginally higher in diabetics than in the healthy controls, but this difference was not statistically significant (Fig. 77). Furthermore, no differences emerged between the various groups of diabetics. Serum cholesterol levels and thrombus length and weight were statistically related in patients with diabetes mellitus, but not in control subjects. The calculated correlation coefficient r for thrombus length and the serum cholesterol level was 0.3484 ($P < 0.01$) and for thrombus weight $r = 0.3779$ ($P < 0.01$).

Thrombus formation time

There was a tendency for the whole blood (WB) thrombus formation time to be shorter in diabetics than in controls but this was not statistically significant (Fig. 78). Furthermore there were no significant differences between complicated and uncomplicated diabetics, nor between complicated vascular and complicated "non-vascular" diabetics. Plasma thrombus formation time, however, was significantly shorter in diabetic patients than in control subjects ($P < 0.001$), but again there were no significant differences between the various categories of diabetics (Fig. 79).

Neither in control nor diabetic subjects was there any significant correlation between whole blood or plasma thrombus formation time on the one hand, and the plasma fibrinogen and serum cholesterol levels on the other.

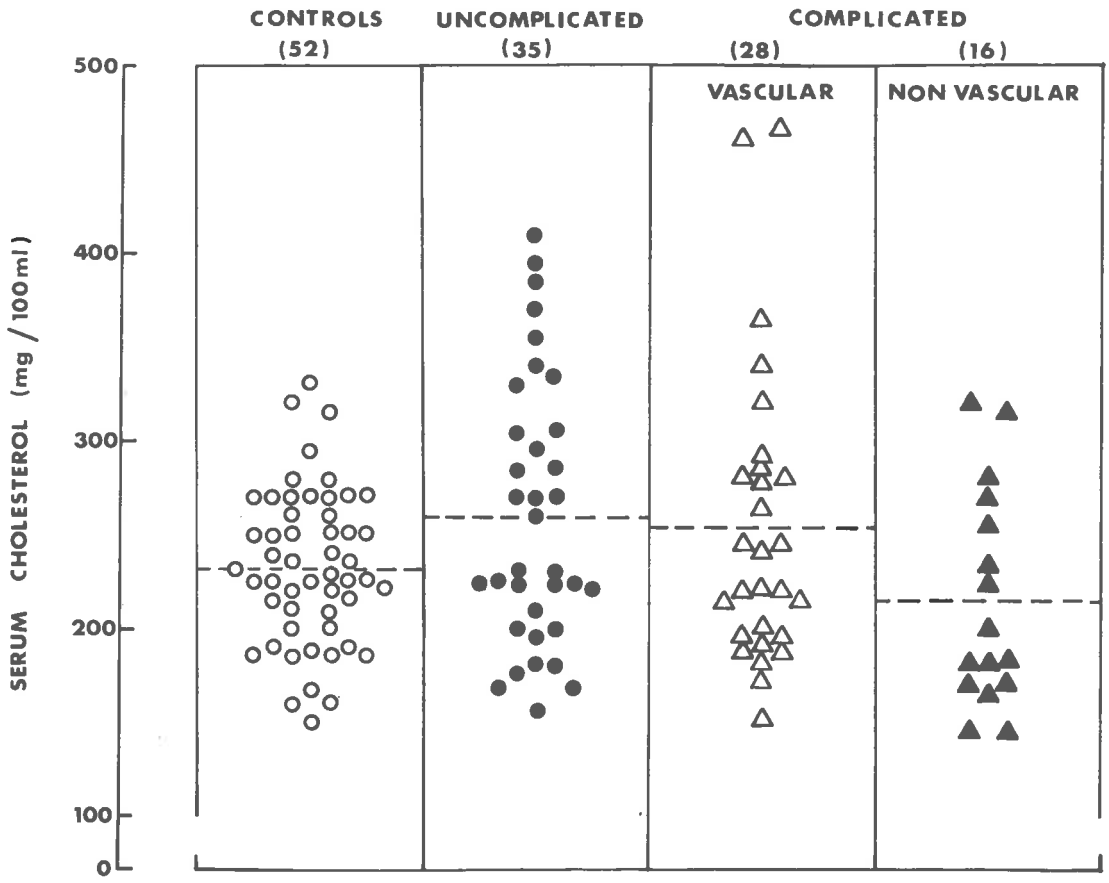


Fig. 77. Total serum cholesterol levels in control subjects, and in the three categories of patients with diabetes mellitus.

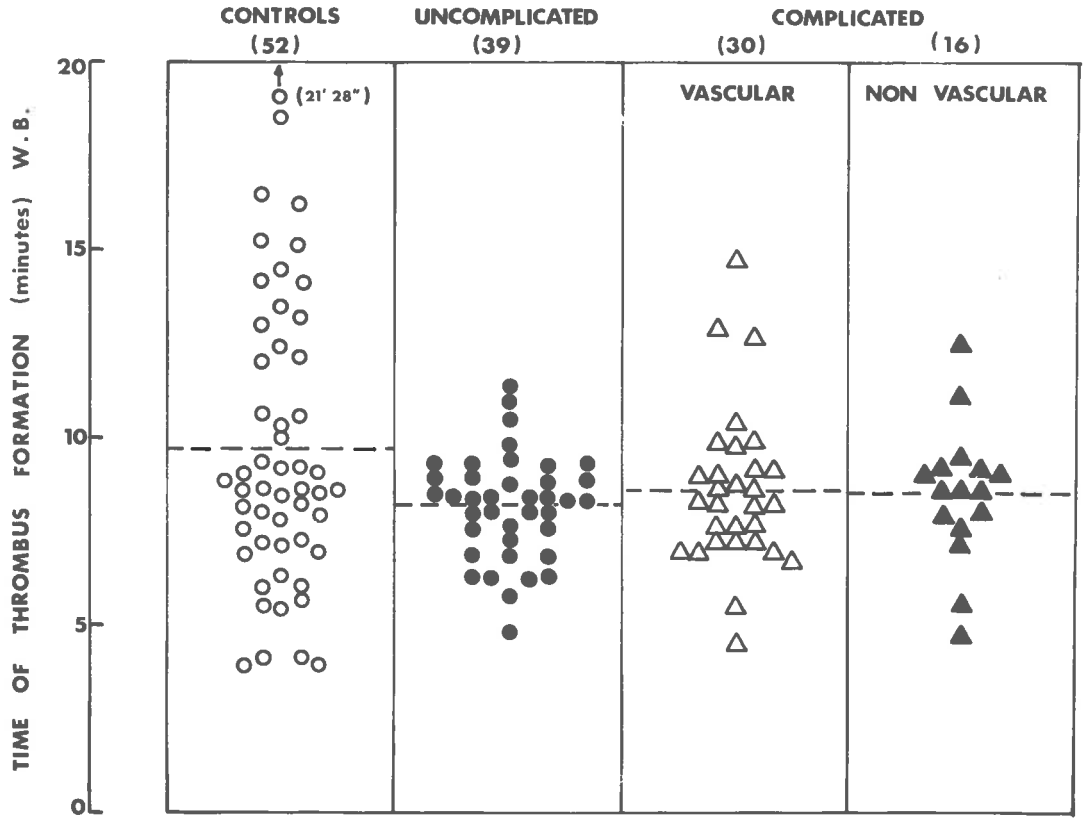


Fig. 78. Mean thrombus formation time (whole blood) in control subjects and in the three categories of patients with diabetes mellitus.

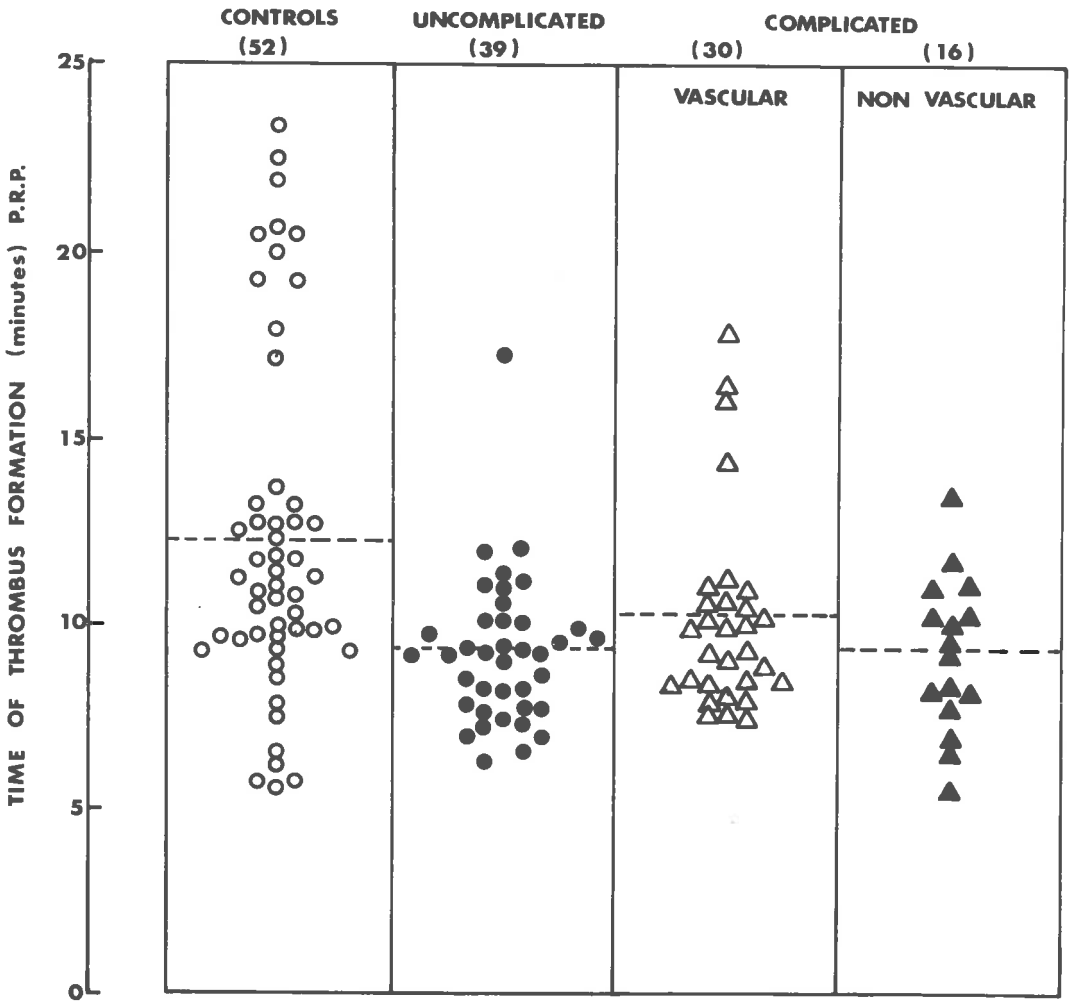


Fig. 79. Mean thrombus formation time (platelet-rich plasma) in control subjects and in the three categories of patients with diabetes mellitus.

TABLE 16

FREQUENCY OF ABNORMAL SPONTANEOUS PLATELET AGGREGATION

Subjects		No. of patients	Days studied	No. of abnormal days	Total platelet abnormality (%)
Controls		52	303	13	4.3
Uncomplicated diabetes		39	166	12	7.2
Complicated diabetes	Vascular	30	119	30	25.2
	Non-vascular	16	69	17	24.6

Frequency of abnormal spontaneous platelet aggregation in healthy control subjects, and in patients with diabetes mellitus. The diabetics are divided into those without overt clinical complications, and those with complications are further subdivided into vascular and non-vascular categories as detailed in the text.

Platelet-rich plasma

In healthy control subjects abnormal spontaneous platelet aggregation occurred on only 4.3% of the 303 days studied. The findings in patients with diabetes mellitus were in sharp contrast. Abnormal spontaneous platelet aggregation was observed in the diabetics with an overall frequency of 16.7% of 354 days studied. Of this, persistence accounted for 9.6% and the snowstorm phenomenon for 7.1%. Table 16 shows the percentage frequency of days of abnormality in patients with uncomplicated and complicated diabetes. The overall frequency of abnormal spontaneous platelet aggregation in the uncomplicated group was 7.2% of the 166 days studied, persistence accounting for 4.2% and the snowstorm phenomenon for 3.0%. In diabetic patients complicated by vascular disease, abnormal spontaneous platelet aggregation occurred on 25.2% of 119 days studied. Persistence occurred on 16.8% and the snowstorm phenomenon on 8.4% of days. The frequency of platelet abnormality in the "non-vascular" group was 24.6% of the 69 days studied (persistence 10.1% and snowstorm phenomenon 14.5%).

Thus the overall frequency of abnormal spontaneous platelet aggregation in patients with diabetes mellitus was some four-fold that of control subjects. The overall frequency of abnormal spontaneous platelet aggregation in complicated diabetics was some six-fold that of controls, and three times greater than in diabetics without overt

TABLE 17

FREQUENCY OF ABNORMAL SPONTANEOUS PLATELET AGGREGATION RELATED TO TREATMENT.

Subjects	Treatment	Days studied	No. of abnormal days	Total platelet abnormality (%)
Healthy controls	-	303	13	4.3
Uncomplicated diabetes mellitus	Diet/oral hypoglycaemics	69	7	10.1
	Insulin	93	5	5.4
Complicated diabetes mellitus	Diet/oral hypoglycaemics	80	20	25.0
	Insulin	108	26	24.1

Frequency of abnormal spontaneous platelet aggregation in patients with diabetes mellitus, considered in relation to the presence or absence of complications, and the type of treatment given.

complications.

The frequency of abnormal platelet behaviour has also been considered in relation to the severity of the diabetes. Once again the type of treatment has been used as an arbitrary index of severity. There was no marked difference in the frequency of platelet abnormality within either the complicated or uncomplicated diabetic sub-groups when related to treatment with diet/oral hypoglycaemic agents, or with insulin therapy (Table 17).

An obvious correlation which could not be performed because of inadequate data is an analysis of the relation between blood glucose levels and platelet behaviour. Regrettably insufficient blood glucose estimations are available at this time to permit any statistical analysis. It is likely, in view of the observations to be related in the next section, that either blood glucose levels or alternatively the level of insulin may relate to platelet behaviour in vivo.

Some preliminary observations on the effects of intravenous glucose on platelets will now be briefly discussed.

The effect of glucose infusions on circulating platelet levels

The effect of a glucose infusion (25 g. glucose in 50 ml. sterile distilled water) on the circulating levels of platelets previously labelled with chromium^{51} was determined in eight healthy subjects. Technical difficulties occurred in one study and this subject has been excluded from the series. In each subject there was a variable but generally

TABLE 18

THE EFFECT OF INTRAVENOUS GLUCOSE ON THE WHOLE BLOOD PLATELET COUNT AND PLATELET RADIOACTIVITY

Subject	% fall in platelet count	% fall in platelet radioactivity	% change blood glucose
T.W.	33	18	280.0
W.T.	31	20	205.0
J.H.	36	3	282.0
I.C.	19	18	529.4
R.McT.	20	10	300.0
K.D.	50	23	230.4
G.G.	6	25	335.7

Effect of intravenous glucose on the whole blood platelet count and platelet radioactivity, and the percentage change in the blood glucose level in seven healthy control subjects.

substantial fall in the whole blood platelet count. The changes, which varied in individuals, are summarized in Table 18. The maximum fall in platelet count occurred 5 minutes after the completion of the infusion in each case. The individual performing the platelet counts was unaware of the nature of the experiment. In three subjects the preinfusion count was restored at 45 minutes. In four subjects, however, despite a gradual increase in platelet count at both 20 and 45 minutes, preinfusion levels had not been attained.

The infusion of glucose intravenously was also associated with a fall in the level of circulating platelet radioactivity. This fall was maximal at 5 minutes and was followed by a gradual increase in subsequent levels of platelet radioactivity (Fig. 80). In only two of the seven subjects were preinfusion levels of platelet radioactivity reached by 45 minutes. It is of interest to note that the percentage fall in platelet radioactivity was usually not as marked as the percentage fall in whole blood platelet count (Table 18).

Surface counting

No detectable alteration in the count rate using external scintillation counters placed over either liver, lung or spleen was observed following the infusion of glucose coincident with the fall in both platelet count and platelet radioactivity.

Other infusion studies

Intravenous glucose tolerance tests were similarly performed on

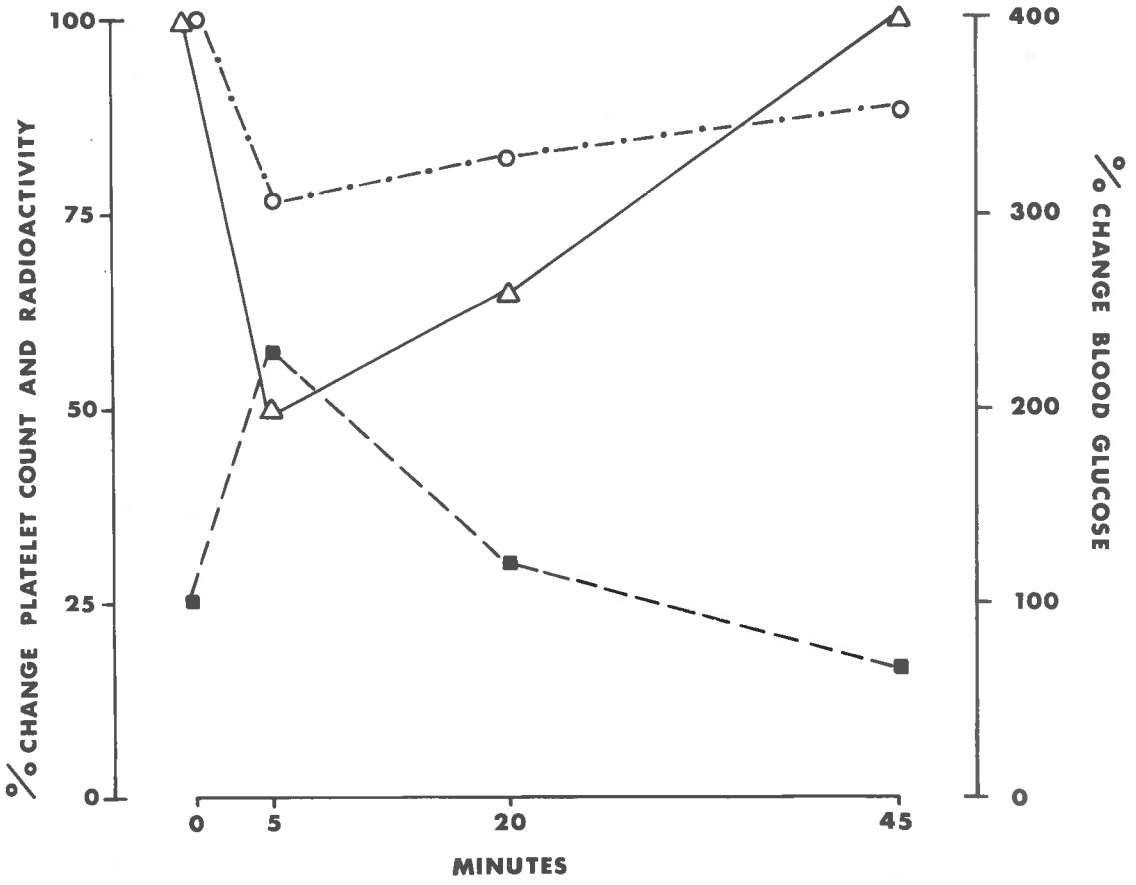


Fig. 80

A representative study showing the percentage change in blood glucose level (■), the percentage change in whole blood platelet count (▲), and platelet radioactivity (○) plotted against time in minutes, after intravenous glucose infusion.

TABLE 19

Nature of study	Pre-infusion	5 min	20 min	45 min	Maximum % change blood glucose
Intravenous glucose	100	81.6	104.0	104.0	288.9
	100	68.4	73.7	84.2	294.1
	100	81.8	86.4	86.4	294.1
	100	91.2	97.1	100.0	289.5
	100	83.5	94.7	97.5	282.4
	100	89.3	107.1	100.0	346.7
	100	82.6	96.3	100.9	328.6
	100	200.7	97.9	97.9	217.6
	100	106.0	101.5	97.7	294.1
Intravenous fructose	100	104.5	100.0	95.5	180.0
	100	95.8	95.8	91.7	158.8
	100	97.5	94.5	102.5	158.8
	100	89.7	-	95.5	152.9

Effects of intravenous glucose and intravenous fructose on the whole blood platelet count expressed as a % of the preinfusion level at the times indicated. All subjects were healthy adults.

a further nine healthy subjects without the prior labelling of platelets with chromium^{51}. A fall in the circulating whole blood platelet count occurred in seven of the nine subjects (Table 19). At 5 minutes this fall in platelet count ranged from 8.8% to 31.6%.

Twenty-five grammes of intravenous fructose was administered to four healthy subjects and samples of blood taken as for the intravenous glucose tolerance tests. In three of the four subjects fructose did not cause a significant fall in the whole blood platelet count. In these cases the change in blood glucose levels was less marked than in those patients receiving a direct infusion of glucose (Table 19). In a fifth subject the experiment was abandoned because of fructose intolerance associated with collapse, sweating, pallor and abdominal pain.

3. Discussion

Using the Chandler apparatus as an in vitro technique it has been demonstrated that thrombus size (weight and length) is significantly increased in patients with diabetes mellitus. The thrombi formed in this system were both longer and heavier in the diabetic population than in control subjects. Similarly the blood of patients with myocardial infarction (experiment 7) and also patients in the postoperative period (experiment 9) is associated with the propensity to form longer and heavier thrombi. In each instance, including the diabetics, thrombus size has shown a significant correlation with the plasma fibrinogen level.

The ultimate significance of the greater thrombus size in each of these situations is uncertain. Certain lipid fractions are known to influence thrombus formation in the Chandler apparatus (Connor and Poole, 1961); the addition of long chain saturated fatty acids was shown to result both in longer thrombi, and a striking reduction in the thrombus formation time. Unsaturated fatty acids had little or no effect. It was suggested that the longer thrombi produced by the "active" fatty acids might be due to the fact that the conversion of fibrinogen to fibrin had been altered in some way resulting in a looser, more strung-out configuration. A similar mechanism might operate in the formation of the longer and heavier thrombi found in patients with diabetes mellitus, particularly as both quantitative and qualitative alterations in lipid metabolism are known to occur in this condition (Bierman et al., 1957). It is therefore not without interest that a significant correlation between thrombus weight and length on the one hand and the total serum cholesterol level on the other was obtained in this study of diabetic patients.

However, plasma fibrinogen levels and thrombus size showed a significant relationship, both in the healthy control subjects as well as in the diabetics, and it would appear likely that the dominant factor determining the longer and heavier thrombi in diabetics as in patients after myocardial infarction is the elevated plasma fibrinogen level found in this condition.

Using the type of treatment as an arbitrary index of disease

severity the possibility that this factor might also be related to thrombus size has been explored. In Table 15 the mean thrombus weight in complicated and non-complicated diabetic categories is considered in relation to type of treatment. It can be seen that within both groups, the type of treatment did not influence the results.

The studies have indicated that the plasma fibrinogen level is significantly elevated in patients with diabetes mellitus. Others (MacKay and Hume, 1964) have not found the plasma fibrinogen level to be elevated in diabetics. In another study investigating the whole blood viscosity of diabetic patients (Skovborg et al., 1966) it was found that the mean plasma fibrinogen level was higher in diabetics than in controls ($P < 0.001$) - a finding which parallels this study. Although the elevated plasma fibrinogen levels might be explained on the basis of tissue damage in the complicated group of diabetics, the reason for the higher values in those with uncomplicated diabetes is less certain. The possibility exists that in these patients, despite the absence of overt clinical complications, tissue damage associated with micro-angiopathic lesions could well be present.

In this study abnormal platelet behaviour was detected on one or more days in 15.4% of control subjects, 23.7% of uncomplicated diabetics, 48.3% of diabetic patients with vascular complications and 50.0% of diabetics whose complications were predominantly "non-vascular". Abnormal platelet aggregation was clearly an episodic

phenomenon, not only in this survey but also in patients with myocardial infarction (experiment 7) and in patients studied during the postoperative period (experiment 9). Because of this episodic behaviour of the platelets, the need for a number of days of study is obvious.

The reasons for this abnormal platelet aggregation in diabetes mellitus are at present speculative. It is possible that the abnormal platelet behaviour detected in the Chandler apparatus using blood from diabetic subjects is related both to the effect and the extent of tissue breakdown. A similar pattern of abnormal platelet behaviour has emerged for patients with myocardial infarction and for patients in the postoperative period in the current studies, findings which add credence to this possibility. Whether these abnormalities are related to a thrombotic tendency in these patients needs clarification, but it is certainly a distinct possibility.

The role of fibrinogen in platelet aggregation has previously received some attention. Hampton and Mitchell (1966b) studied the electrophoretic mobility of human platelets from patients with pneumonia, myocardial infarction and after surgical operations and found an increase in the sensitivity of platelets to both ADP and nor-adrenaline. Although they found that the time of onset of the changes may parallel the rise in plasma fibrinogen levels, the fibrinogen levels remained elevated for longer than abnormal platelet behaviour could be detected. From experiments on rabbits' blood

(McLean, Maxwell and Hertler, 1964) it was concluded that fibrinogen plays a part in platelet aggregation, and recently (Inceman et al., 1966), from the results of studies of platelet aggregation in patients with congenital afibrinogenaemia, it was concluded that fibrinogen is essential for platelet spreading on glass, ADP-induced platelet aggregation, but only when the concentration of ADP is small, and in the late stages of metamorphosis. Because of the possibility albeit disputed, that fibrinogen may be a co-factor in platelet aggregation the percentage frequency of abnormal platelet aggregation was related to the plasma fibrinogen level in both diabetic and control subjects and a significant correlation was demonstrated for diabetic patients ($P < 0.01$) but not for controls.

The thrombus formation time showed a tendency to be shorter in diabetics than in controls but the exact nature of the changes which lead to this reduction are uncertain. Once again abnormalities of lipid metabolism must be considered. It is known that long chain saturated fatty acids accelerate blood clotting in certain in vitro systems (Poole, 1955; O'Brien, 1957). Furthermore, they also cause shortening of the thrombus formation time in the Chandler apparatus (Connor and Poole, 1961), and it has been postulated that these effects are mediated by the activation of the Hageman factor (Margolis, 1962). It is possible that this mechanism might contribute to the shorter thrombus formation time noted in the diabetics in this

study. It was not possible to demonstrate any significant correlation between thrombus formation time and the total serum cholesterol but this does not preclude the possibility that other qualitative or quantitative lipid abnormalities may have contributed to the shorter thrombus formation time.

Bridges and colleagues (1965) studied the degree of platelet stickiness in patients with diabetes mellitus using the technique of McDonald and Edgill (1958). They claimed an increased stickiness in diabetics, as compared with normal subjects. Moreover, the administration of glucose, either by mouth or intravenously apparently resulted in an increased platelet stickiness both in controls and in diabetics. These findings are consistent with the present infusion studies in normal subjects, in whom a fall in platelet count after intravenous glucose was observed. This fall could reflect an increased platelet adhesiveness or stickiness.

The fall in circulating platelet levels following the infusion of glucose was also obvious when levels of circulating platelet radioactivity were estimated in conjunction with platelet counts. The percentage fall in platelet radioactivity was not as marked as the overall fall in direct platelet count. There is evidence that there may be at least two populations of platelets in circulation (Webber and Firkin, 1965) and that younger platelets are more adhesive than older platelets (Wright, 1942; Hirsh et al., 1966). It is possible that the greater fall in direct platelet count after glucose might reflect the

loss of the younger and more adhesive platelets during the labelling procedure, or their failure to re-appear in the circulation as viable units after the platelet infusion.

Surface counting data did not reflect any particular organ as the site of segregation of platelets from circulation after glucose loading. The reasons for this may be twofold: platelets may have been removed from the circulation within the vascular tree throughout the body; or the fall in platelet radioactivity being less marked than the fall in platelet count, was of insufficient magnitude for increases in organ activity to be detected by the apparatus used.

Experiment 9: In vitro thrombosis and platelet behaviour after operation

There is a tendency in the postoperative period for patients to suffer the complications of thrombo-embolism. The role of the blood platelet in thrombus formation is well recorded and its behaviour in the postoperative period has been explored by a number of workers. Hueck in 1926 was the first to record a rise in the blood platelet count after operation and his findings were subsequently confirmed by Dawbarn et al., in 1928. Dawbarn and colleagues also noted that the rise in postoperative platelet count was most marked between the twelfth and fourteenth postoperative days. Wright (1942) noted that the postoperative and post-partum rise in platelet

count was associated with a ready capacity for platelets to adhere to glass.

Using the Chandler apparatus as an in vitro model the blood of patients after operation has been examined in an attempt to determine whether there is abnormal spontaneous platelet aggregation and thrombosis in these circumstances. An attempt has been made to relate the frequency of abnormal thrombosis and platelet aggregation to the extent of the operation. In addition several platelet survival studies have been carried out.

1. Materials and Methods

The Chandler apparatus was used to study both recalcified citrated whole blood and platelet-rich plasma of patients in the postoperative period. This technique has been fully described in experiment 7.

Platelet labelling

Platelets from 300 ml. blood were labelled with radioactive disodium chromate ($Na_2Cr^{51}O_4$) using the method of Davey and Lander (1963), resuspended in plasma and reinjected within 2½ hours of the initial collection of blood (see experiment 8).

Platelet survival

After injection of platelets, samples of venous blood were collected from the other arm at frequent intervals for 4 hours and

TABLE 20

Age distribution of the postoperative subjects studied, compared with controls. Postoperative subjects are subdivided into those with an operation score of 3 or less and those with a score of 4 or more (see text).

Subject categories	Age group (years)						
	11-20	21-30	31-40	41-50	51-60	61-70	71
Controls	3	9	12	15	10	3	-
Postoperative							
score ≤ 3	4	6	3	4	7	6	6
score > 3	-	-	-	5	7	7	8
Total postoperative	4	6	3	9	14	13	14

TABLE 21

The score index, indicating operations with a score of 3 or less and those with a score of 4 or more.

Score ≤ 3	Score > 3
Biopsy	Amputation Leg
Lumbar Sympathectomy	Femoro-Popliteal Bypass
Cholecystectomy	Insertion Femoral Pin
Hysterectomy	Radical Mastectomy
Varicose Vein Stripping	Bowel Resection
Uterine Repair Operation	Aortic Aneurysm Repair
Appendicectomy	Prostatectomy

at daily intervals thereafter for 10 to 12 days.

Subjects studied

Fifty-two healthy control subjects and 63 patients who had had an operation performed were investigation (Table 20). Postoperative patients were subdivided into two categories according to the extent of the operation. Using the scoring system outlined by Moore and Ball (1952) patients were grouped into those with an operation score of 3 or less and those with a score of 4 or more. An indication of the score index is shown in Table 21. This is an arbitrary index only and the score may vary in individual patients depending on the conditions at operation.

An attempt was made to study patients for a minimum of 5 days because of the episodic nature of platelet abnormalities which had been noted in similar investigations carried out in patients with myocardial infarction and diabetes mellitus.

The 52 healthy controls were studied for a total of 303 days and the 63 postoperative patients for a total of 300 days.

2. Results

Fig. 81 shows the mean thrombus length (mm.) and the mean thrombus weight (mg.) in healthy control subjects and in patients in the postoperative period. Thrombus length and weight were significantly greater in postoperative patients than in controls ($P < 0.001$ in each case). Thrombus length and weight did not differ significantly

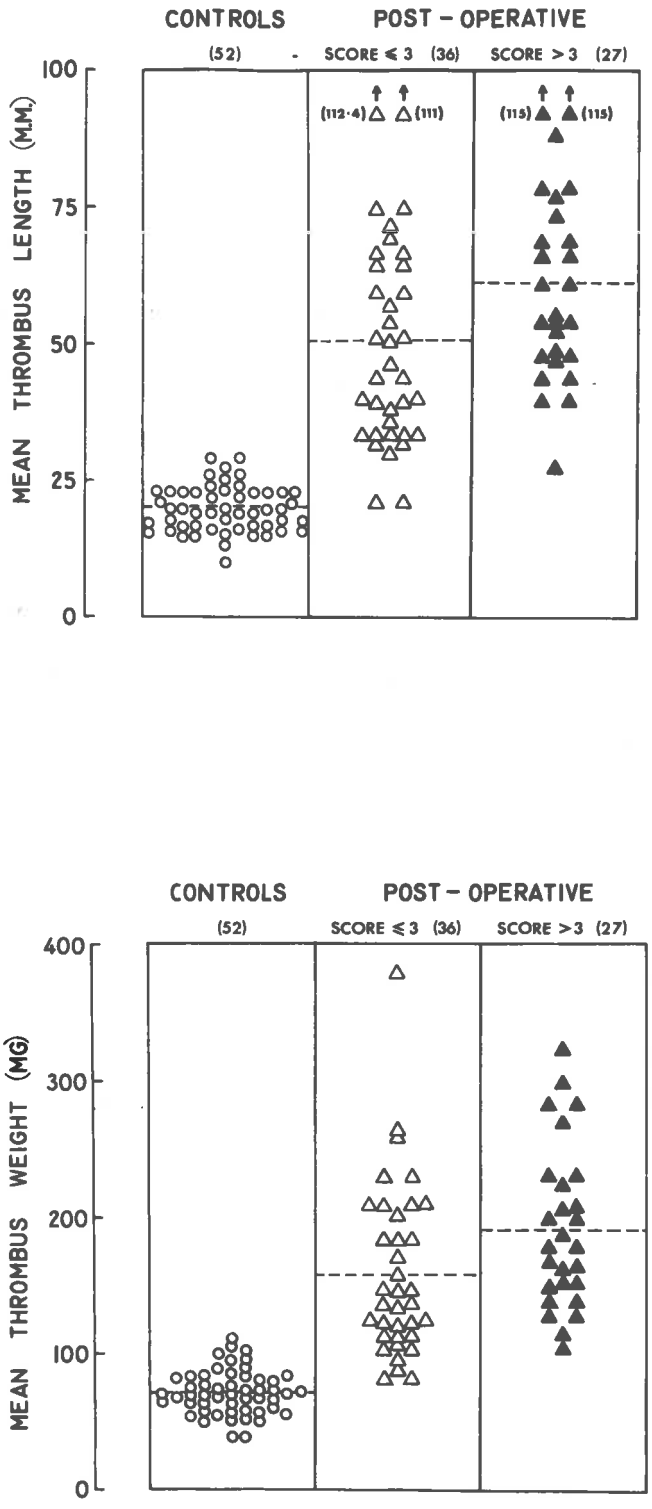


Fig. 81. Mean thrombus length (TOP) and weight (BOTTOM) in controls and in the two categories of post-operative subjects.

in those patients with an operation score greater than 3 and those with a score of 3 or less.

Plasma fibrinogen

Plasma fibrinogen levels were considerably elevated in patients in the postoperative period (Fig. 82). There was a significant difference between the plasma fibrinogen levels of controls and of postoperative patients ($P < 0.001$). Furthermore the difference between major and minor operation groups was also significant as plasma fibrinogen levels were considerably higher in those whose operation was more extensive ($P < 0.01$).

Plasma fibrinogen and thrombus size

Both in control and in postoperative subjects there was a statistically significant correlation between the plasma fibrinogen and thrombus length. In controls the calculated correlation coefficient r was 0.4952 ($P < 0.001$) and in patients after operation r was 0.5397 ($P < 0.001$). Similar correlations emerged for thrombus weight and plasma fibrinogen, but there was no correlation between thrombus size and the serum cholesterol level. Serial plasma fibrinogen estimations were carried out in 14 patients for the duration of the study. Once again the plasma fibrinogen levels paralleled changes in the whole blood thrombus length and weight (Fig. 83).

Thrombus formation time

The whole blood thrombus formation time showed no significant

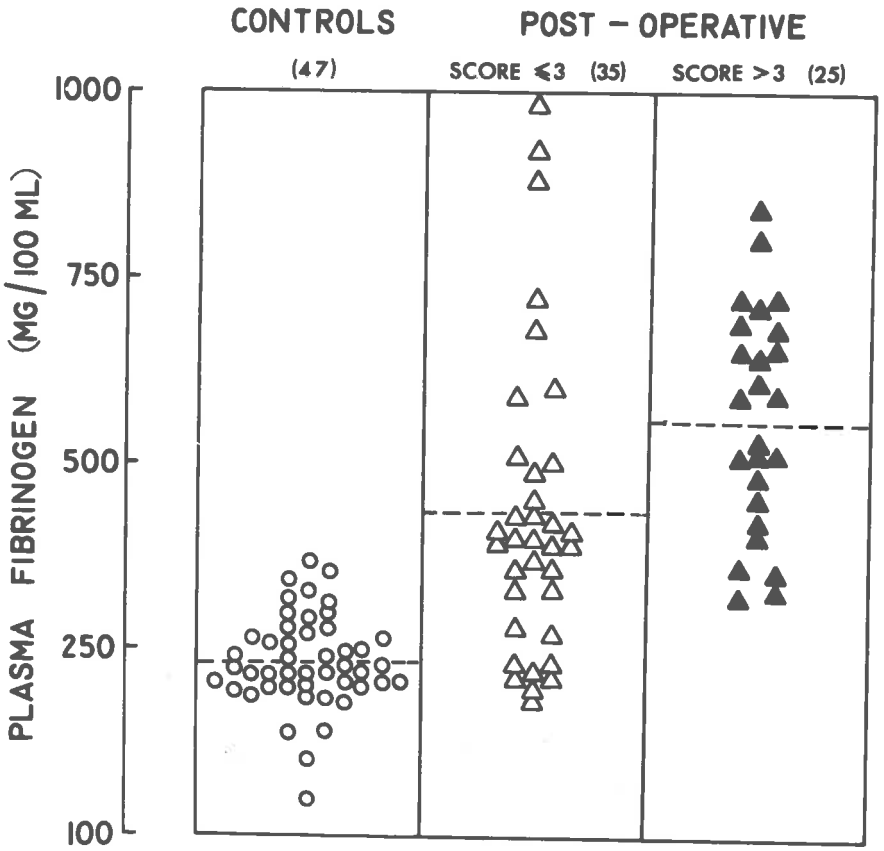


Fig. 82. Plasma fibrinogen level in control subjects and in the two categories of post-operative patients. The number of subjects in each category is indicated in brackets at the top of each column.

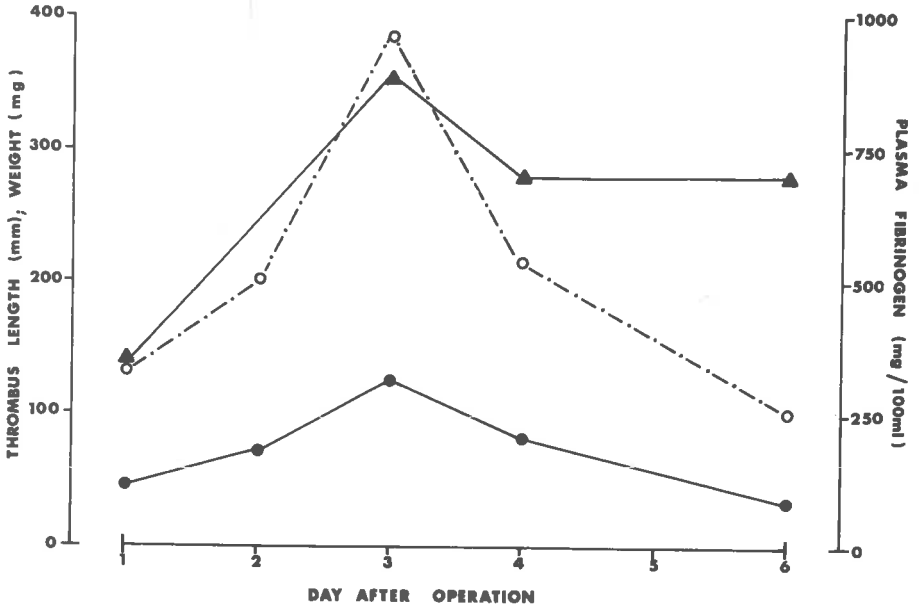


Fig. 83a

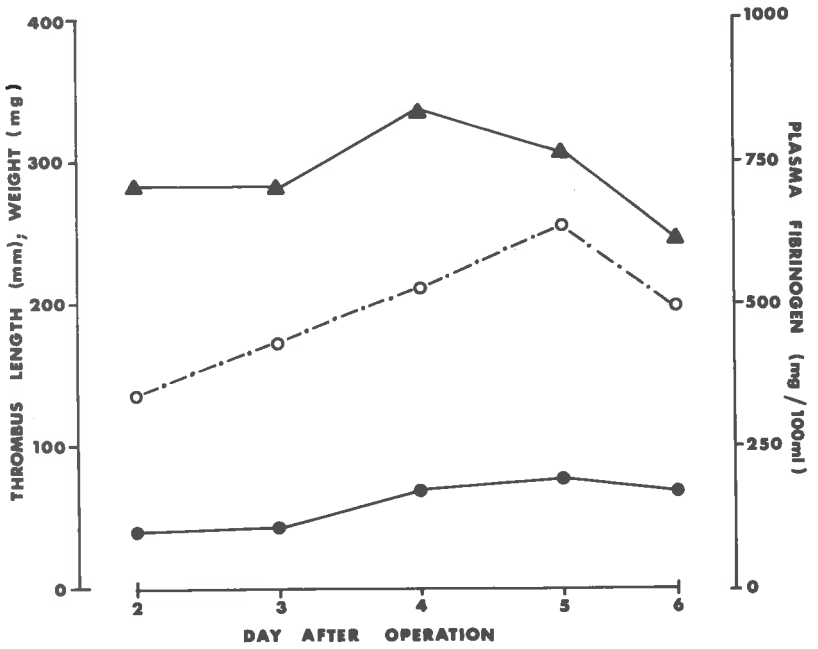


Fig. 83b

Serial plasma fibrinogen levels (▲) in two patients (a: B191; b: B186) in parallel with the changes in thrombus length (●) and weight (o).

TABLE 22

Frequency of abnormal spontaneous platelet aggregation in control subjects and in the two post operative categories considered separately and together.

Subjects	Number of subjects	Days studied	Persistence (%)	Snowstorm Phenomenon (%)	Total platelet abnormality (%)
Controls	52	303	3.0	1.3	4.3
Post operative					
Score $\leqslant$ 3	36	151	19.2	14.6	33.8
Score $>$ 3	27	149	20.1	18.1	38.2
Combined	63	300	19.7	16.3	36.0

difference between postoperative patients and control subjects. Neither was there a statistically significant difference between whole blood thrombus formation time in each of the postoperative subgroups (Fig. 84). The platelet-rich plasma thrombus formation time was shorter in patients after operation than in controls ($P < 0.01$). Further, the mean thrombus formation time of those with operation scores of 3 or less was significantly shorter ($P < 0.05$) than those with a score of greater than 3 (Fig. 84).

Platelet rich plasma

In healthy control subjects abnormal spontaneous platelet aggregation was present on only 4.3% of the total days studied. After operation abnormal platelet aggregation occurred on 36% of the 300 days studied. Persistence occurred on 19.7% of these days and the snowstorm phenomenon on 16.3%. Table 22 shows the percentage frequency of abnormal platelet aggregation in each of the postoperative subgroups. In patients with an operation score of 3 or less the frequency of platelet aggregation was 33.8% of the 151 days studied. Persistence occurred on 19.2% and the snowstorm phenomenon on 14.6% of these days. In those with an operation score of greater than 3, abnormal spontaneous platelet aggregation occurred on 38.2% of the 149 days of study. Persistence accounted for 20.1% and the snowstorm phenomenon for 18.1% of the abnormalities.

Thus abnormal spontaneous platelet aggregation was some nine

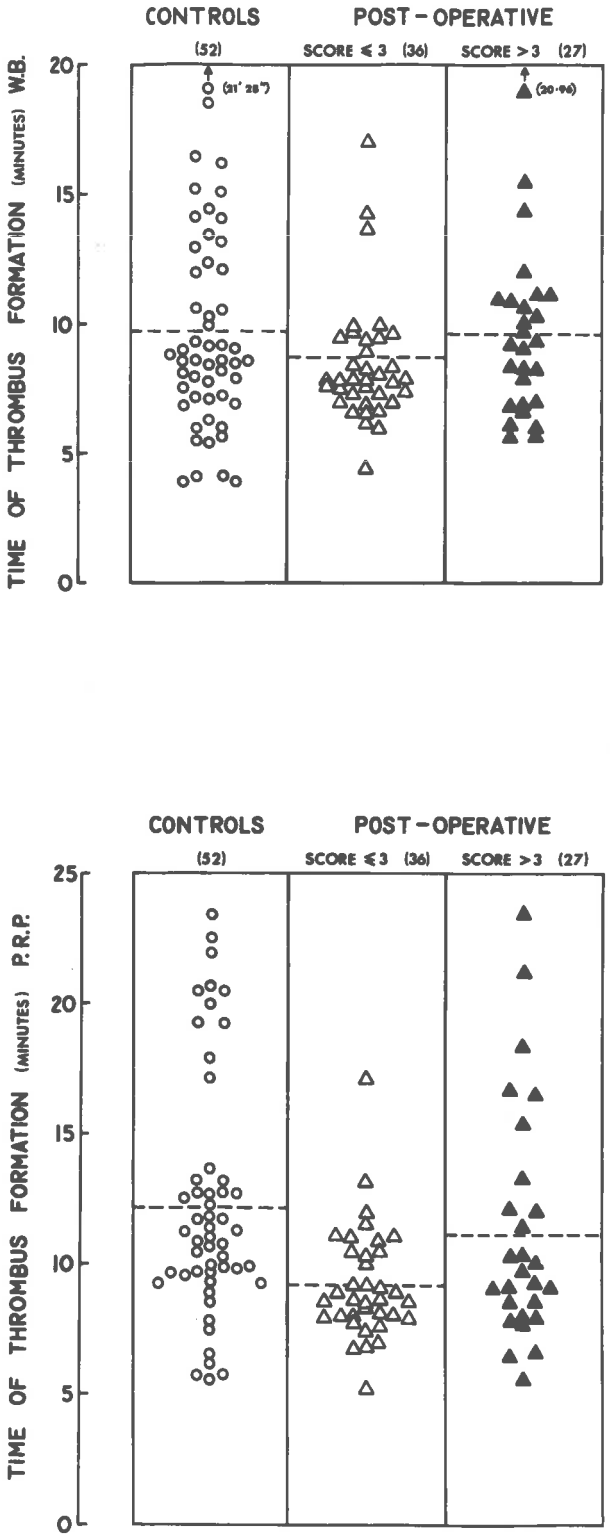


Fig. 84. Thrombus formation time for whole blood (TOP) and plasma (BOTTOM) in controls and in the two post-operative categories.

times more common among patients after operation than among healthy controls.

Platelet survival studies - preliminary observations

Platelet survival studies were carried out on 5 patients in the post operative period. In 2 the pattern of survival was normal and in 3 platelet survival was reduced (Fig. 85). In Figure 86 two patients (O.G. and W.B.) are depicted, in whom both in vitro abnormalities using the Chandler technique, and reduced platelet survival were recorded. It is of special interest that in patient O.G. a snowstorm phenomenon was observed 2 days prior to the development of clinical thrombosis in the leg veins. Further, in patient W.B., both in vitro studies and platelet survival studies were normal at 5 months after operation.

Time after operation

Results were also analysed with respect to the time after operation. The mean values for thrombus length and weight on each of the days after operation are shown for two patients in Fig. 83. There was a gradual increase in thrombus length and weight with the passage of time after operation. Thrombus formation time (WB and P.R.P.) was not affected by the time after operation. Although there was little change in the overall frequency of abnormal spontaneous platelet aggregation with the passage of time after operation, there was some alteration in the relative frequency of the persistence and the snowstorm phenomena. In the early post operative period, persistence occurred more

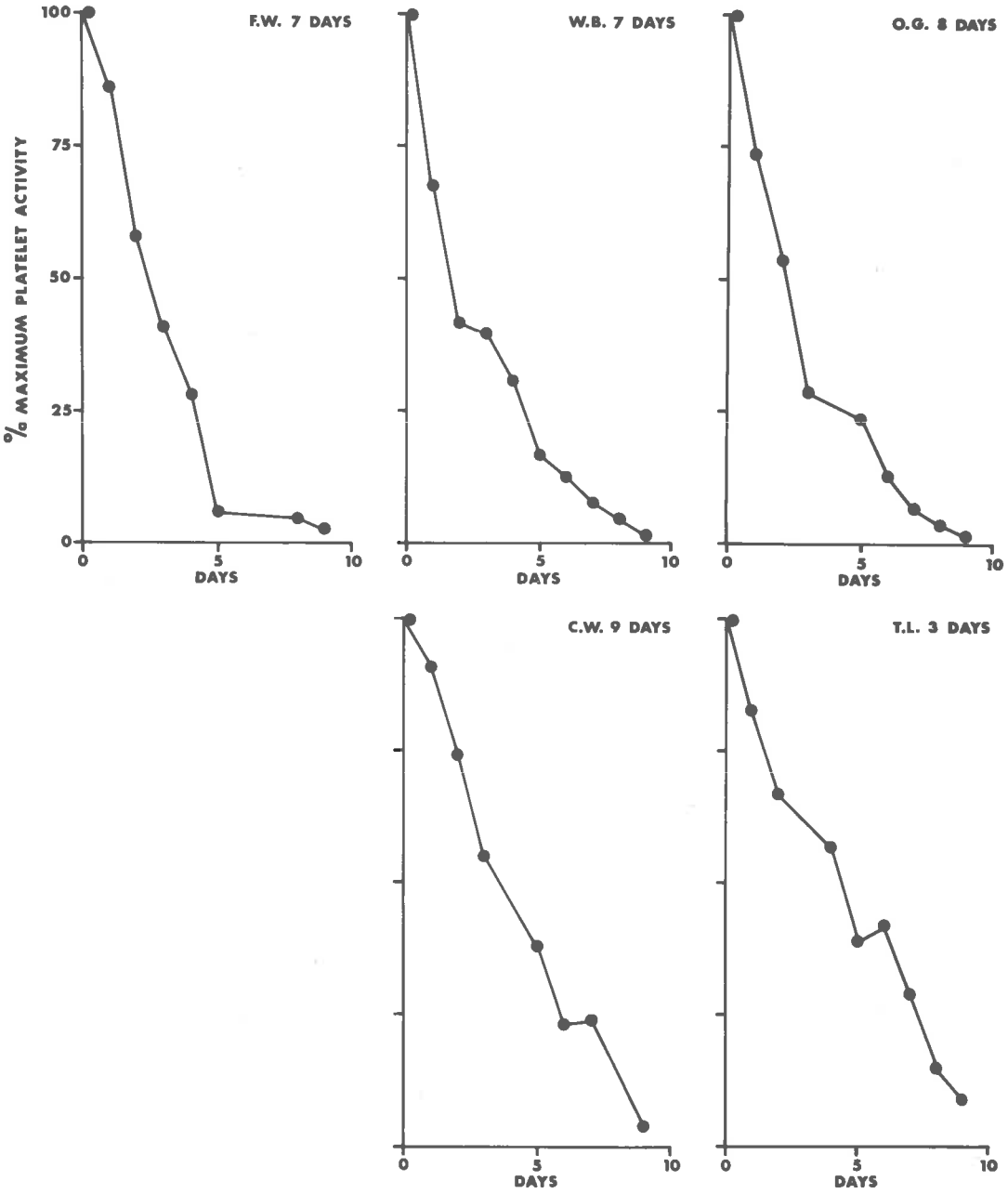


Fig. 85. Platelet survival curves of five post-operative subjects, showing the percentage maximum platelet activity plotted against time. In three patients (F. W.; W. B.; and O. G.) platelet survival was reduced, while in the other two (C. W. and T. L.) the pattern of survival was normal. In each instance the number of days after operation at which the study was commenced is indicated.

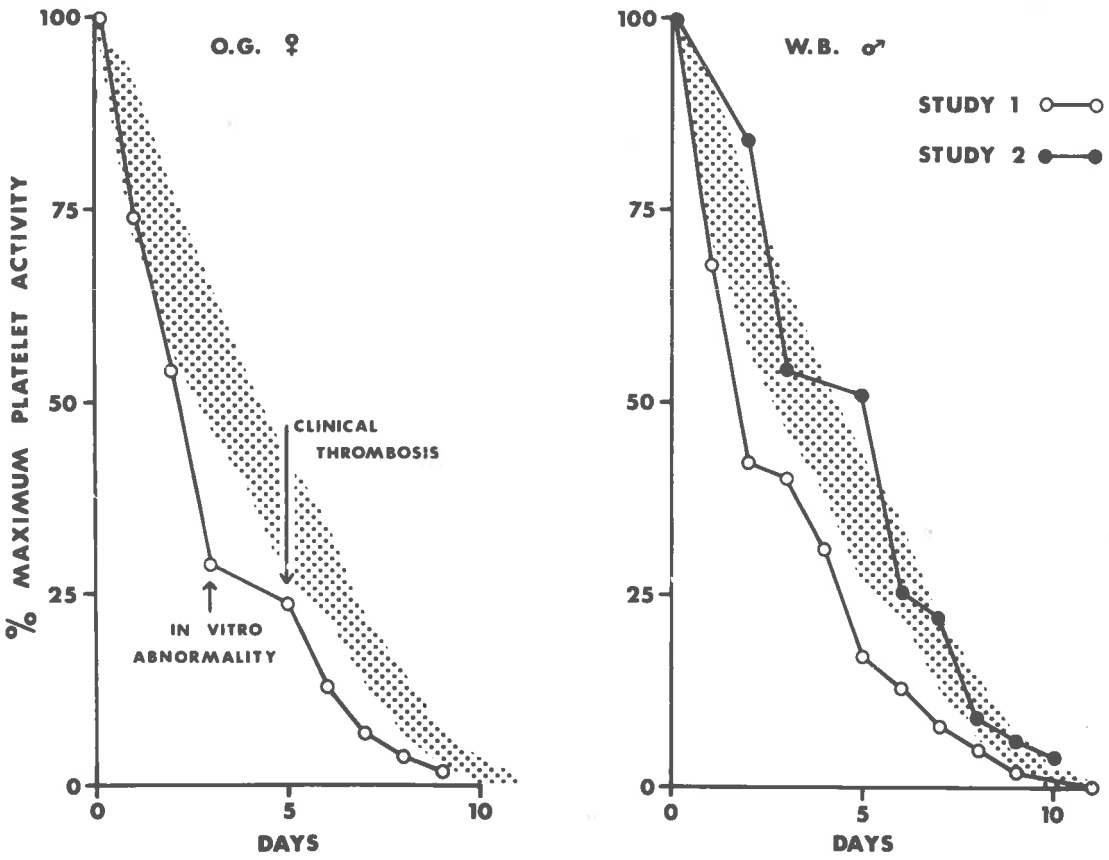


Fig. 86. Reduced platelet survival in two patients in the post-operative period. In patient O. G. a snowstorm phenomenon was first observed 12 days after operation and 2 days prior to the development of clinical thrombosis in the leg veins. Patient W. B. was first studied 7 days after operation, and showed a snowstorm phenomenon throughout the course of this study; at the same time a reduced platelet survival was found (o). This patient was studied again 5 months later, and both in vitro studies and platelet survival studies (●) were normal.

commonly than the snowstorm phenomenon. As the time after operation increased there was a tendency for persistence to occur less commonly and for the snowstorm phenomenon to become more frequent (Fig. 87).

Vascular disease v. non-vascular disease

Results were also analysed according to the presence (22 subjects) or absence (41 subjects) of vascular disease in the patients who had had an operation. There was a tendency for the thrombi to be both longer and heavier in those with vascular disease and operation than in those without vascular disease. These differences were not statistically significant.

There was no difference in the mean total serum cholesterol levels in either group but the plasma fibrinogen level was higher in those with vascular disease. Once again, however, this difference was not statistically significant. Abnormal spontaneous platelet aggregation occurred on 35.6% days in those with non-vascular disease and on 35.1% of days in those with vascular disease. Persistence occurred on 23.4 and 11.7% of days in non-vascular and vascular disease respectively. The snowstorm phenomenon occurred on 12.2 and 23.4% of days in non-vascular and vascular subgroups respectively.

DISCUSSION

Using the Chandler apparatus it has been demonstrated that the blood of patients after operation forms thrombi which are significantly longer and heavier than those of healthy control

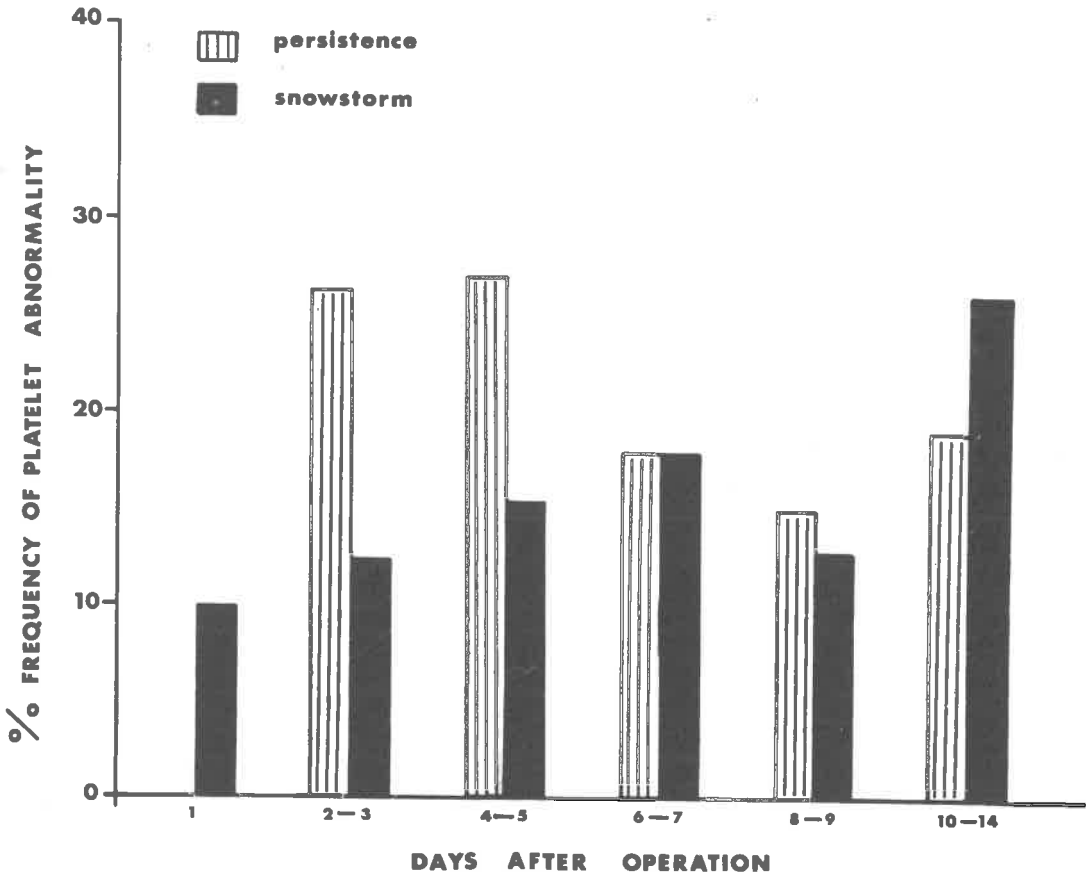


Fig. 87. Showing a tendency for the % frequency of persistence to occur less commonly and for the snowstorm phenomenon to become more frequent with the passage of time after operation.

subjects. As in the patients with myocardial infarction and with the diabetic subjects there was a significant correlation between thrombus size and plasma fibrinogen levels ($P < 0.001$). This relationship between plasma fibrinogen and thrombus size was reinforced by observations in post operative subjects in whom serial daily fibrinogen levels were carried out after operation.

The results show a significantly greater frequency of abnormal spontaneous platelet aggregation in post operative patients than in healthy controls. Abnormal platelet behaviour was detected on 36.0% of the days studied and was clearly an episodic event. The extent of operation had no apparent effect on the incidence of platelet abnormality as there was little difference in its frequency in patients with an operation score of 3 or less and those with a score greater than 3. Emmons and Mitchell (1965) demonstrated a clearcut change in platelet clumping activity after surgical operations. This increase in the in vitro response of platelets to ADP, ATP, 5H-T and nor-adrenaline followed a similar time course to the increased adhesiveness described by Wright (1942) and to the incidence of clinical thrombo-embolism. The precise nature of the change in platelet behaviour and its possible relation to plasma or platelet factors has remained speculative.

Hampton and Mitchell (1966b) studied the electrokinetic response of human platelets to ADP and nor-adrenaline in a variety of acute illnesses including the post operative state. They found a maximum increase in sensitivity on the first

post operative day in all cases, followed by a gradual return to the preoperative levels. The increase in platelet sensitivity after minor operations was of shorter duration than that of patients undergoing more extensive operation. The time of onset of these changes may have paralleled the rise in plasma fibrinogen levels but this was an unlikely explanation for the increased platelet sensitivity in these subjects as the fibrinogen level frequently remained elevated for longer than increased platelet sensitivity was manifest. In the Chandler study there was no correlation between abnormal platelet behaviour and plasma fibrinogen level.

This Chandler study has shown two distinct patterns of abnormal spontaneous platelet aggregation - the persistence phenomenon and the snowstorm phenomenon. In patients in the post operative period the early phases are characterized by a relatively greater frequency of the persistence phenomenon than the snowstorm phenomenon. With the passage of time after operation the frequency of the persistence phenomenon became less and the snowstorm increased. This raises the question as to whether the technique is measuring different degrees of the same phenomenon or whether these are two entirely separate reactions with no real inter-relation?; at present this is not clear, although the former seems more likely.

In the platelet survival studies two subjects had a normal platelet survival and three a reduced platelet survival. In these patients platelet survival was most abnormal in subjects whose

mean platelet count was highest. This may indicate that increased platelet utilization in these subjects was well compensated and had been matched by either increased production or release of platelets from megakaryocytes or by mobilization from other platelet pools or depots. The decrease in platelet survival with a shift of the curve to the left is compatible with an increased random removal from circulation. This may be accounted for by increased utilization during the haemostasis and thrombosis of vessels damaged during the course of operation, and possibly accentuated by the release of tissue factors, for example adenosine diphosphate or tissue thromboplastin which promote platelet clumping. Furthermore, as a result of operation a large proportion of young platelets may be released into circulation to meet the increased demands. There is increasing evidence that young platelets are more adhesive than others (Wright, 1942; Hirsch et al., 1966) and with a young population, a proportion of those which survive handling in vitro and circulate as viable units will be more readily lost.

The reduction in platelet survival in these patients in the post operative period may be a further reflection of an abnormal tendency to thrombosis in these individuals.

PART V

SUMMARY (including an indication of wherein the thesis is considered to have advanced medical knowledge)

PART V - SUMMARY

The conclusions drawn from each experiment have been detailed part by part. It remains to recapitulate the contents of this thesis, and to indicate wherein the results have contributed to medical knowledge.

In part I a review of thrombosis was presented. In scope, it was directed to information broadly relevant to the experiments performed, and includes publications on the subject to December, 1966.

The principal results and conclusions of the various experiments are:

PART II (i) In normocholesterolaemic rabbits the organisation of "thrombi" rich in platelets results in the production of arterial plaques possessing all of the features classically enumerated by Rindfleisch as characteristic of the atheromatous plaques in the arteries of man (Fig. 88). It should be emphasised that lipid bearing lesions can develop by the organisation of thrombi in the absence of any dietary manipulation.

(ii) It was concluded that the phagocytosis of platelets, and probably also polymorphonuclear leucocytes were the major sources of foam cell lipid in plaques.

(iii) The infiltration of thrombi by polymorphonuclear leucocytes appeared to be related in effect and extent to the platelet content of the thrombi, and this could

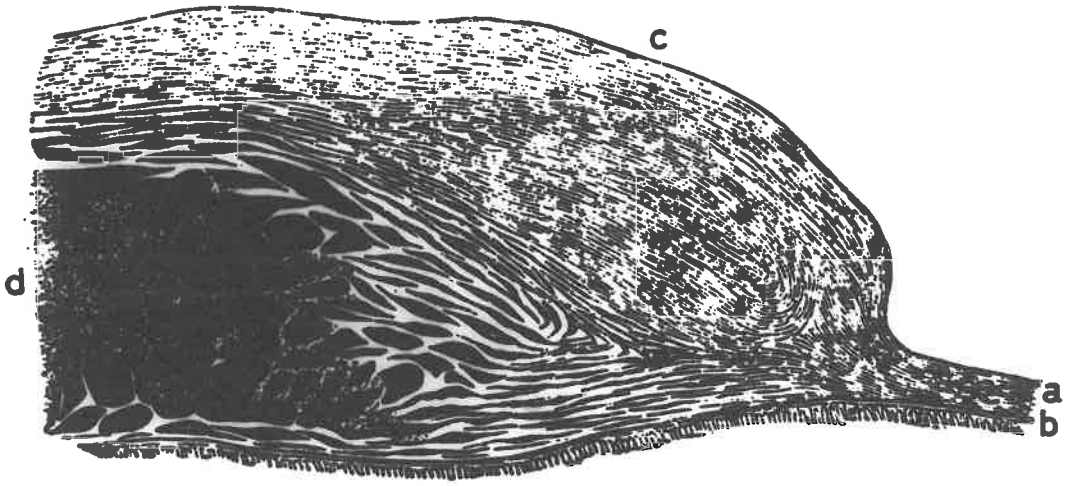


Fig. 88. Diagram of a classical atherosclerotic plaque from Rindfleisch (1872). The original legend reads: "Chronic endoarteritis. Section through an indurated elevation of the inner coat, which is already undergoing fatty degeneration in its interior. b. Junction of inner and middle coats; a. Inner coat; At c this is hyperplastic and thickened, while at d it has become converted into an atheromatous abscess."

be explained by the release of a leucotactic factor from platelets. This latter property may be of fundamental importance in the resolution of thrombi and haemostatic plugs and would account for the characteristic disposition of leucocytes around the margins of platelet clumps.

(iv) At the cellular level, thrombi are organised and incorporated into the vessel wall by endothelialisation and by infiltration with endothelial cells and with circulating white cells. The endothelial cells are the only cells of the vessel wall to participate in organisation.

(v) Hypercholesterolaemia enhances the lipid content of the plaques. The lipid-rich plasma trapped within the emboli was considered to be an important source of the additional lipid in plaques. The escape of lipid from young capillaries in the organising thrombi could also contribute to plaque lipidisation.

(vi) Dietary induced hypercholesterolaemia and lipaemia was associated with impaired thrombolysis, and enhanced plaque calcification.

(vii) Circulating foam cells were incorporated into the vessel wall in the same manner as the emboli. There was also evidence of incorporation of lipid

TABLE 23

Summary of results of experiments in part IV. Comparisons between the various categories of patients on the one hand and control subjects on the other are shown. Differences within categories are not shown - for these latter comparisons refer to text.

VARIABLES AND CORRELATIONS MEASURED	PATIENT CATEGORY		
	MYOCARDIAL INFARCTION	DIABETES MELLITUS	POST OPERATIVE
1. Mean thrombus length	+	+	+
2. Mean thrombus weight	+	+	+
3. Plasma fibrinogen	+	+	+
4. Total serum cholesterol	N.S.	N.S.	N.S.
5. Thrombus formation time (WB)	+	N.S.	N.S.
6. Thrombus formation time (PRP)	+	-	-
7. Abnormal platelet aggregation	+	+	+
8. Fibrinogen and thrombus size	+	+	+
9. Cholesterol and thrombus size	N.S.	+	N.S.
10. Fibrinogen and thrombus formation time	N.S.	N.S.	N.S.
11. Cholesterol and thrombus formation time	N.S.	N.S.	N.S.
12. Fibrinogen and abnormal platelet aggregation	N.S.	+	N.S.
13. Abnormal platelet aggregation and thrombus formation time	N.S.	N.S.	N.S.

(continued)

TABLE 23 (continued)

+ = significant increase or correlation respectively.

- = significant decrease.

N.S. = no significant difference or no correlation respectively.

into the vessel wall by means other than the endothelialization of foam cell aggregates.

PART III(viii) The three catecholamines, adrenaline, nor-adrenaline and dopamine enhanced platelet aggregation produced by the nucleotides ADP or ATP, and abolished the inhibitory action of adenosine, AMP, ATP and ADP on nucleotide induced aggregation.

(ix) Catecholamine potentiation of nucleotide activity was further enhanced by the saturated fatty acid sodium stearate.

(x) The three methyl xanthines, caffeine, theobromine and aminophylline were found to prevent and reverse the platelet aggregation produced by the combined effect of catecholamine and nucleotide. These compounds also inhibited and reversed platelet aggregation induced by ADP.

The experiments detailed in part III were all performed in vitro.

PART IV (xi) The findings are summarised in Table 23. The main findings were: that the fibrinogen level in the three categories of patients studied was greater than in control subjects, and that this was associated with a greater weight and length of "thrombus" in the Chandler apparatus; that the plasma thrombus formation time in the Chandler apparatus was shorter

in diabetic and post operative subjects than in control subjects; and that spontaneous platelet aggregation occurred more frequently in platelet-rich plasma samples from the three categories of patients than in samples from control subjects.

(xii) The infusion of intravenous glucose was found to reduce the whole blood platelet count in healthy subjects, a reduction which was not associated with any specific organ uptake of Cr^{51} labelled platelets.

One possible interpretation of the data available on the subject of thrombosis and arterial disease is that a primary alteration occurs in the circulating blood, and that this results in the formation of thrombi which then organise into wall plaques. The studies contained in this thesis were directed at the solution of some of the problems arising from this proposal, and some of their aims appear to have been achieved.

The deposition of a thrombus and its evolution into an atherosclerotic plaque as proposed by Duguid still is not generally accepted as the principal or a significant mechanism in the genesis or growth of an atherosclerotic plaque. The observations in part II indicate that the organisation of a thrombus can lead to the development of an atheromatous plaque. Moreover, the presence of lipid in the plaque can be explained by this mechanism alone. On the other hand, these observations do not indicate in what way

atherosclerosis begins, and it may be that it begins as some intimal lipid derangement and that thrombosis is a subsequent complication thereby increasing the size of a plaque.

The total serum cholesterol level seems to be the best predictor of coronary heart disease but this statistical association does not necessarily imply a causal role. The observations in experiment 2 (Part II) suggest a link between hypercholesterolaemia and arterial disease. Firstly, the artificial thrombi produced with blood from cholesterol-fed rabbits contained significantly more platelets, and perhaps this is a reflection of enhanced platelet aggregation in these animals. The demonstration (experiment 5) of an effect of sodium stearate on platelet aggregation is not without interest in this respect. Secondly, plaques resulting from the organisation of thrombi contained significantly greater quantities of lipid in hypercholesterolaemic animals than in normocholesterolaemic animals.

The findings in part IV could be of help in attempting to understand the mechanisms involved in causing thrombotic complications particularly in diabetic and post operative subjects. The results indicate that there may be a generalised change in the circulating blood, in the nature of an increased "stickiness" of platelets. The whole blood thrombus formation time was significantly longer in subjects after myocardial infarction, but did not differ between control subjects and patients with diabetes

mellitus or patients after operation. In the latter two categories of patients, however, the plasma thrombus formation time was significantly shorter than in control subjects. These apparently conflicting results with plasma and whole blood are difficult to evaluate, whereas the evidence in favour of abnormal platelet behaviour in the various categories studied is clear cut and this suggests that a generalised change in the circulating blood may exist and that this is due to increased platelet "stickiness" rather than hypercoagulability. However, these conclusions are presented with reservation.

Finally, the experiments have supplied some preliminary clues as to what factors may be influencing platelet behaviour. These include lipids (sodium stearate, and cholesterol or other lipids associated with hypercholesterolaemia), glucose, catecholamines and platelet age. The verification of these postulates requires much further study however. In particular, the author has considered in some detail, the possible role of the catecholamines in platelet adhesion and aggregation in the haemostatic process, and in thrombosis. A number of assumptions for which experimental proof is as yet lacking were made in

proposing an explanation for the effects of catecholamines on platelet aggregation. Such speculation finds ample justification in the words of Robert Browning -

"Ah, but a man's reach should exceed his grasp,

Or what's a heaven for?"

APPENDICES

APPENDIX A

METHOD OF IMBEDDING

Paraffin		Carbowax	
Number of blocks	Number of slides	Number of blocks	Number of slides
2,909	20,967	185	266

Total number of blocks - 3,094

Total number of slides - 21,233

Showing number of blocks of lung tissue processed in paraffin and in carbowax and the number of slides prepared from these blocks.
A minimum of two sections were mounted on each slide.
Hence approximately 42,000 sections were prepared and examined in this study.

APPENDIX B

CONTROL SUBJECTS

Expt. no.	Name of subject	Age	Sex
A1	WHITE, T.	44	M
A2	ROBERTSON, I.	38	M
A3	ADDISON, M.	41	M
A4	HANSEN, M.	45	M
A5	HARDY, E.	55	M
A6	IBBOTSON, R.	38	M
A7	HOWARTH, W.	40	M
A8	HARDY, D.	42	M
A9	BANKS, A.	47	M
A10	TAYLOR, A.	42	F
A11	SHEPPARD, R.	42	M
A12	DIXON, S.	37	F
A13	KINLOUGH, R.	28	F
A14	GLEW, G.	27	M
A15	DAVIS, P.	37	M
A16	LUKE, C.	23	M
A17	DERRINGTON, W.	39	M
A18	CLAPP, K.	38	M
A19	DAVIES, G.	49	M
A20	LLOYD, J.V.	25	M
A21	MITCHELL, N.	43	F
A22	GORMLY, G.	47	M
A23	HACKETT, W.	44	M
A24	NOLAN, W.	49	M
A25	NEWMAN, D.	45	M
A26	LOOKE, E.	28	F
A27	BASTEN, A.	25	M
A28	BARTA, J.	38	M
A29	WATTS, P.	57	M
A30	IRVING, E.	37	F
A31	KLEINE, J.	26	M
A32	MURRELL, T.	31	M
A33	MATTSCHOSS, K.	19	M
A34	HOWELL, D.	23	M
A35	MEYER, G.	20	M
A36	HOOPER, H.	19	M
A37	GORMLEY, B.	22	M

APPENDIX B (cont.)

Expt. no.	Name of subject	Age	Sex
A38	PAIN, R.	32	M
A39	ADDIS, A.	37	M
A40	EDWARDS, J.	59	M
A41	NICHOLLS, J.	62	M
A42	MARTIN, C.	55	M
A43	LLOYD, J.F.	64	M
A44	GITTINS, W.	62	M
A45	JACKSON, H.	58	M
A46	CHADWICK, E.	57	M
A47	ROCCA, F.	56	M
A48	SCALZI, P.	53	M
A49	DORSETT, A.	50	M
A50	SILVESTRI, P.	57	M
A52	BAIN, H.	50	M
A53	HERON, A.	58	M

APPENDIX C

PATIENTS WITH MYOCARDIAL INFARCTION

Expt. no.	Name of patient	Hospital unit record (U.R.) no.	Age	Sex	Time after myocardial infarction
B3	McKEOGH, M.	002015	80	F	1 day
B4	EDWARDS, W.	049335	57	M	20 days
B5	STACEY, M.	048277	64	F	38 days
B6	MULLINER, V.	050627	71	F	7 days
B8	ROGERS, F.	051153	57	M	5 days
B9	FRANKLIN, B.	051886	69	M	10 days
B10	BOWLEY, K.	004404	54	M	3 years**
B11	WATERS, A.	016943	73	F	11 days
B13	BENNETT, W.	052855	66	M	3 days
B15	CRUMMEY, T.	053399	58	M	6 days
B16	HARVEY, R.	053387	59	M	14 days
B19	WOODCOCK, A.	057658	72	F	6 days
B27	VARGA, T.	062818	54	M	18 days
B29	PASH, G.	064860	54	F	15 days*
B30	ABEL, W.	037839	63	M	16 days
B35	PEARCE, M.	068003	55	M	5 days
B36	KAS, E.	035162	45	M	10 months
B37	FINDLAY, R.	059460	56	M	4 months
B38	BADMAN, L.	067845	75	M	5 days
B39	WELLER, L.	-	63	M	6 days
B40	ALLEN, I.	068368	66	F	2 days
B41	WADDLE, A.	000125	69	M	2 days
B43	CARRAGHER, M.	068310	43	M	10 days
B44	WINTON, E.	046250	62	M	7 days
B45	TILL, R.	023727	75	M	7 days*
B47	RAGLESS, O.	068987	54	M	8 days
B48	RUSH, C.	022892	57	M	2 days
B50	NOBLE, C.	051336	68	M	9 months**
B51	DEVERELL, A.	069258	50	M	9 days
B52	TILLEY, H.	068527	70	M	1 month
B53	SWANN, B.	069715	39	M	4 days
B54	HOFFMANN, M.	069149	70	F	2 days
B55	FLETCHER, H.	035296	78	M	3 weeks
B56	LEE, A.	-	54	M	7 days

APPENDIX C (cont.)

Expt. no.	Name of patient	Hospital unit record (U.R.) no.	Age	Sex	Time after myocardial infarction
119	McDONALD, F.	030513	61	M	4 years**
121	HEATLEY, A.	005499	53	F	4 weeks
123	CORBELL, S.	042006	62	M	6 days
124	JOHNSTONE, A.	034492	54	M	3 months
126	GOODGER, O.	028269	57	F	? years
128	CULVILL, G.	043647	51	M	6 years**
129	KARAVENKAS, J.	043253	57	M	17 days
133	BALL, D.	044008	50	M	21 days
134	MARTENS, H.	045065	55	M	12 days
136	FARSON, A.	044416	49	M	4 weeks
145	STANLEY, E.	032822	65	M	7 months
153	BULLEN, W.	011190	64	M	9 years
174	FREEMAN, G.	001412	54	M	? years
176	DODD, T.	060990	16	M	4 weeks
179	MOLLER, W.	058097	61	M	4 months
181	PAVLOVICH, W.	065934	67	M	9 days

* Died during the course of study.

** More than one episode of myocardial infarction.

APPENDIX D

PATIENTS WITH DIABETES MELLITUS

Expt. No.	Name of patient	Hospital unit record (U.R.) no.	Age	Sex	Treatment*	Complications**
B5	STACEY, M.	048277	64	F	1	++
B61	LANGLANDS, G.	008841	74	M	1	++
B62	ADCOCK, C.	057138	83	M	1	++
B63	MONTELEONE, N.	071038	42	F	2	+
B64	STANDISH, E.	070998	77	F	1	++
B65	HARVEY, P.	026170	71	M	1	++
B76	SWANDALE, D.	010452	60	F	2	++
B77	O'DONNELL, V.	072347	67	F	1	++
B79	FERGUSON, W.	029958	67	M	2	++
B81	SMITH, M.	000767	67	F	1	Nil
B82	HERDE, E.	012705	73	F	2	Nil
B83	SOUTER, A.	069256	66	M	1	Nil
B85	THOMAS, D.	056180	63	M	2	++
B86	LAUNER, H.	057681	62	M	2	Nil
B87	EDWARDS, T.	067000	21	M	2	+
B88	WILLIAMS, G.	001016	51	F	1	++
B89	DEUTROM, E.	074824	50	M	2	++
B90	CHAPLIN, E.	074636	44	M	2	++
B91	PHELAN, E.	016019	68	M	2	+
B92	SMITH, E.	075039	46	M	2	+
B94	KRAMER, F.	071361	48	M	2	++
B95	SANSOM, H.	075362	76	M	1	++
B96	HEYNEN, C.	075352	56	M	2	+
B97	MILLIGAN, D.	002129	59	M	2	+
B98	EDGE, G.	075387	59	M	2	Nil
B99	WESTERN, R.	068499	73	M	1	Nil
B100	HARRIS, F.	075865	44	M	2	+
B102	BOOTH, R.	075817	63	F	1	+
B103	WILLIS, O.	076540	64	M	2	++
B104	WOOD, D.	076882	66	F	2	++
B105	HORSBURGH, F.	073517	65	F	2	++
B106	PATTERSON, D.	043407	71	F	1	++
B107	PANNELL, B.	027016	44	F	2	++
B108	ANDERSON, K.	027268	45	M	2	Nil

APPENDIX D (cont.)

Expt. no.	Name of patient	Hospital unit record (U.R.) no.	Age	Sex	Treat-ment*	Complica-tions**
B109	HORE, S.	076524	72	F	1	Nil
B110	McNAB, H.	018583	63	F	2	++
B111	RICHARDS, M.	016349	82	F	1	Nil
B117	GATT, S.	077962	68	M	2	Nil
B118	DONISE, L.	082952	48	M	1	+
B119	DICKENSON, L.	000446	62	F	2	Nil
B120	BURCHELL, A.	083600	51	M	1	Nil
B122	GEORGE, M.	083344	70	M	1	++
B123	PETTMAN, E.	083111	69	F	2	Nil
B124	BUTTON, J.	083798	81	M	1	Nil
B127	CLINTON, E.	083794	23	M	2	Nil
B130	PARMITER, V.	040087	39	F	2	++
B131	HENNESSY, K.	084768	69	M	1	Nil
B133	MARAFIOTI, T.	001388	51	F	1	++
B135	WEBSTER, F.	085306	71	M	1	++
B136	TALBOT, A.	078164	71	M	1	Nil
B137	HAMBLEN, L.	085341	17	F	2	Nil
B138	ROBERTS, J.	010230	80	M	1	++
B140	GYE, J.	085908	62	M	1	+
B141	BARNETT, E.	081522	83	M	1	++
B142	HEWISH, E.	017946	70	F	2	Nil
B143	BASTIAN, D.	085604	30	M	2	Nil
B144	STEPHENS, K.	085609	43	M	2	++
B145	QUEALE, H.	001326	50	M	2	Nil
B146	KONARSKI, J.	074820	49	M	1	Nil
B147	FEWINGS, E.	086176	68	M	1	++
B148	KAPPLER, P.	010807	71	M	1	Nil
B150	MYNOTT, W.	082185	70	M	1	Nil
B152	WEMAN, S.	086650	64	M	1	+
B153	McINERNEY, S.	073724	69	M	1	+
B154	von der BORCH, A.	083866	62	F	2	Nil
B155	JENKINS, C.	049704	56	M	2	Nil
B157	HARRIS, L.	002022	68	F	22	Nil
B158	STONE, F.	002587	82	F	2	Nil
B159	COTIS, S.	007087	22	F	2	Nil

APPENDIX D (cont.)

Expt. no.	Name of patient	Hospital unit record (U.R.) no.	Age	Sex	Treatment*	Complications**
B160	ELDRIDGE, F.	029543	68	M	1	Nil
B161	DUCZECK, J.	087121	65	M	1	++
B162	BROWN, G.	-	17	M	2	Nil
B163	ANTHONY, J.	088310	52	M	1	Nil
B164	WESCOMBE, A.	071065	48	M	2	Nil
B165	MATTHEWS, A.	023450	31	F	2	+
B166	BURNESS, V.	054799	68	F	1	Nil
B167	RIJNBEEK, A.	015923	37	M	2	+
B168	MAXFIELD, E	021194	80	F	2	Nil
B169	CLARKE, E.	038132	78	F	2	+
189	FLAVELL, C.	000447	60	M	1	Nil
195	MONTELEONE, N.	071038	41	F	2	Nil
197	RINGWALDT, A.	066668	45	M	1	Nil
202	CANINO, M.	-	27	F	2	Nil

* Diabetic patients treated with diet and/or oral hypoglycaemics (1) or with insulin therapy (2).

** Diabetic patients with no complications (Nil) or with vascular (++) and "non-vascular" (+) complications (see text).

APPENDIX E

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POST-OPERATIVE PATIENTS

Expt. no.	Name of patient	Hospital unit record (U. R.) no.	Age	Sex	Operation
B7	HILLIER, W.	048266	56	M	Sympathectomy
B12	MALYCHA, L.	049990	60	M	Leg amputation
B17	BUBNER, R.	006714	42	F	Mastectomy
B20	HASLINGDEN, E.	057364	81	F	Bowel resection
B21	STODDEN, M.	059184	46	M	Repair perforated duodenal ulcer
B23	PERROTT, C.	038350	68	F	Cholecystectomy
B24	BROWN, W.	005332	69	M	Herniorrhaphy
B25	McCORD, L.	034430	52	M	Femoral endarterectomy
B26	STUBBS, C.	062181	73	M	Prostatectomy
B28	LACKMAN, C.	065284	17	M	Appendicectomy
B67	HOMANN, D.	071828	22	F	Sympathectomy
B68	McKENZIE, H.	071819	71	F	Laparotomy (pancreatitis)
B69	RUBINO, S.	072054	21	M	Appendicectomy
B70	ARMSTRONG, J.	071821	65	M	Femoro-popliteal bypass
B71	BREYNARD, N.	072192	52	M	Resection aortic aneurysm
B72	HORE, W.	072581	18	M	Appendicectomy
B73	REID, E.	072706	53	M	Appendicectomy
B74	LEWIS, L.	072517	49	F	Femoro-popliteal bypass
B78	BUTLER, A.	026358	45	M	Femoro-popliteal bypass
B80	BLANC, J.	073523	68	M	Resection aortic aneurysm
B84	REIMANN, E.	072476	57	M	Femoro-popliteal bypass
B93	ROBERTSON, A.	064880	73	M	Leg amputation
B114	SMITH, E.	074515	67	M	Cholecystectomy
B115	FITZSIMMONS, D.	078854	51	F	Appendicectomy
B121	KING, T.	009086	21	M	Appendicectomy
B125	HARLOW, K.	084156	23	F	Appendicectomy
B126	HENDERSON, R.	035172	59	M	Leg amputation

APPENDIX E (cont.)

Exp. no.	Name of patient	Hospital unit record (U. R.) no.	Age	Sex	Operation
B128	CLIFT, S.	064524	68	M	Prostatectomy
B129	GILMORE, G.	084308	82	F	Sympathectomy
B139	MUIRSON, D.	085744	61	F	Mastectomy
B141	BARNETT, E.	081522	83	M	Leg amputation
B171	VAUGHAN, A.	089119	50	M	Sympathectomy
B172	CARASARIDIS, C.	089162	38	M	Cholecystectomy
B173	COBB, B.	014933	72	M	Leg amputation
B174	MUNRO, T.	049597	41	F	Hysterectomy
B175	BURZACOTT, M.	078009	63	F	Varicose veins stripped
B176	REYNOLDS, G.	006391	81	F	Cholecystectomy
B177	MILTON, E.	088205	57	F	Cholecystectomy
B178	BYSTER, J.	087865	34	F	Cholecystectomy
B179	NORTON, E.	040295	47	F	Manchester repair
B180	GROVES, B.	019191	39	F	Varicose veins stripped
B181	HART, D.	089747	17	M	Repair fractured femur
B182	GREGOR, J.	078865	83	M	Cholecysto-jejunostomy
B183	WENGER, H.	082690	50	M	Femoro-popliteal bypass
B184	ROBERTS, E.	089663	27	F	Cholecystectomy
B186	FITZPATRICK, A.	008278	86	F	Femoral prosthesis
B187	AHWEN, C.	070855	54	F	Repair uterine prolapse
B188	GREEN, M.	003617	87	F	Biopsy vulva
B189	DIN, F.	091556	71	F	Manchester repair
B190	PLIMMER, F.	090351	71	M	Femoro-popliteal bypass
B191	WARD, L.	092112	53	M	Repair perforated duodenal ulcer

APPENDIX E (cont.)

Expt. no.	Name of patient	Hospital unit record (U. R.) no.	Age	Sex	Operation
B192	HADDON, L.	027005	47	M	Appendicectomy
B193	FARRELLY, T.	088873	64	F	Manchester repair
B194	PITT, M.	006789	25	F	Manchester repair
B195	HARRY, T.	026335	78	M	Herniorrhaphy
B196	DIEPENBROEK, M.	092223	19	M	Varicose veins stripped
B197	HEIGHT, P.	092640	55	F	Biopsy cervix
B198	HARDWAY, R.	062227	69	M	Cholecystectomy and herniorrhaphy
126	GOODGER, O.	028269	57	F	Sympathectomy*
130	BULLEN, W.	011190	64	M	Aortic dacron graft*
132	WICKENS, C.	041124	67	M	Ileo-femoral dacron graft*
149	WALLIS, F.	000409	59	F	Hemicolectomy*
168	LAZDINS, T.	008997	61	M	Femoro-popliteal bypass*

* Platelet survival studies were performed on these 5 patients.

APPENDIX F

Studies contained in this thesis which have been published in journals. (Other papers have recently been submitted for publication.)

1. Ardlie, N. G., Glew, G. and Schwartz, C. J. (1966). Influence of catecholamines on nucleotide-induced platelet aggregation. Nature (Lond.) 212, 415.

2. Ardlie, N. G., Kinlough, R. L., Glew, G. and Schwartz, C. J. (1966). Fatty acids and in vitro platelet aggregation. Aust. J. exp. Biol. med. Sci. 44, 105.

3. Ardlie, N. G., Kinlough, R. L. and Schwartz, C. J. (1966). In vitro thrombosis and platelet aggregation in myocardial infarction. Brit. Med. J. 1, 888.

Various aspects of the work contained in this thesis have been presented at the respective scientific meetings:

i. "Some factors influencing platelet aggregation in vitro." The 10th Annual General Meeting of the College of Pathologists of Australia, Melbourne, August, 1965.

ii. "Observations on the organisation of thrombi." The 11th Annual General Meeting of the College of Pathologists of Australia, Sydney, August, 1966.

iii. "Thrombosis and platelet aggregation in myocardial infarction: Studies using the Chandler apparatus." The Royal Australasian College of Physicians, Annual Meeting, Adelaide, May, 1966.

iv. "Platelet behaviour and in vitro thrombosis in patients with diabetes mellitus." The XIth International Congress of Haematology, Sydney, August, 1966.

APPENDIX F (cont.)

v. "*In vitro* thrombosis and spontaneous platelet aggregation in diabetic subjects." The Australian Society for Medical Research, Adelaide, November, 1966.

APPENDIX G

A list of other published works in the field of cardiovascular research. Additional papers have been submitted for publication.

1. Ardlie, N. G. and Schwartz, C. J. (1965). Arterial pathology in the Australian reptile: A comparative study. J. Path. Bact. 90, 487.

2. Schwartz, C. J. and Ardlie, N. G. (1966). Observations on the natural history of coronary artery disease. Austral. Ann. Med. 15, 101.

3. Prathap, K., Ardlie, N. G., Paterson, J. C. and Schwartz, C. J. (1966). Spontaneous arterial lesions in the Antarctic seal. Arch. Path. 82, 287.

APPENDIX H

The methods employed in tests of equality of means for fibrinogen, mean thrombus length and mean thrombus weight in the three categories of subjects included in the studies in Part IV of the thesis.

MYOCARDIAL INFARCTION

CONTROLS versus TOTAL MYOCARDIAL INFARCTS

1. fibrinogen	Behren's test
2. mean thrombus length	covariance analysis
3. mean thrombus weight	covariance analysis

INFARCTS ON ANTICOAGULANT versus INFARCTS OFF ANTICOAGULANT

1. fibrinogen	t-test
2. mean thrombus length	covariance analysis
3. mean thrombus weight	t-test

DIABETES MELLITUS

CONTROLS versus TOTAL DIABETES MELLITUS

1. fibrinogen	covariance analysis
2. mean thrombus length	covariance analysis
3. mean thrombus weight	covariance analysis

UNCOMPLICATED DIABETICS versus COMPLICATED DIABETICS

1. fibrinogen	no comparison of means possible because regression coefficients unequal.
2. mean thrombus length	covariance analysis
3. mean thrombus weight	covariance analysis

APPENDIX H (continued)

COMPLICATED VASCULAR versus COMPLICATED NON-VASCULAR

1.	fibrinogen	t-test
2.	mean thrombus length	t-test
3.	mean thrombus weight	covariance analysis

POST OPERATIVE

CONTROLS versus TOTAL POST OPERATIVE

1.	fibrinogen	Behren's test
2.	mean thrombus length	covariance analysis
3.	mean thrombus weight	covariance analysis

POST OPERATIVE SCORE ≤ 3 versus POST OPERATIVE SCORE > 3

1.	fibrinogen	Behren's test
2.	mean thrombus length	covariance analysis
3.	mean thrombus weight	covariance analysis

APPENDIX J

Frequency (expressed as a percentage of the number of subjects studied) of the two patterns of abnormal platelet aggregation considered together, in controls, and in patients with myocardial infarction, patients with diabetes mellitus and patients after operation.

SUBJECT CATEGORY	TOTAL PLATELET ABNORMALITY (% SUBJECTS)
Controls	15.4
Myocardial infarct	76.0
Diabetes mellitus	37.3
Post operative	68.3

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