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PLATELET ADHESIVENESS: A possible link between the lipid
and thrombogenic theories of atheroma production.

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S U M M A R Y

THOMAS FYFE.

Platelet Adhesiveness: A possible link between the lipid and thrombogenic theories of atheroma production.

SUMMARY

The main aim of the thesis is the investigation of the relationships between platelet adhesiveness and serum lipids in vivo. If it were possible to link these then the platelet would become a bridge between the lipid and thrombogenic theories of atheroma production.

Section I includes a review of the lipid and thrombogenic theories of atheroma production together with the abundant evidence from the literature linking platelets and lipids in vitro. Evidence that platelets are involved in the formation of atheromatous plaques is also presented.

The lipids measured in the experimental sections of the thesis were plasma non-esterified fatty acids (NEFA), because these are known to affect platelet function in vitro, and serum cholesterol and serum triglyceride which are known to be elevated in subjects with ischaemic heart disease. Blood sugar levels were also measured.

Platelet adhesiveness was used as the measure of platelet function because it is known to be elevated in subjects with ischaemic heart disease and clearly if platelets are important in the development of atheroma, this measure is likely to be the relevant one. A further reason was that the effects of plasma NEFA on platelets in vitro appeared from the literature to be mediated by a mechanism involving adenosine diphosphate (ADP) on which platelet adhesiveness measured by the Payling Wright method also depended.

The various methods of measuring platelet adhesiveness are reviewed and the chosen method, a modification of that of Payling Wright, is described in detail. The accuracy of the method was investigated and a brief study was conducted to determine the variation in platelet adhesiveness due to age, sex and the presence of ischaemic heart disease.

In Section II, studies of platelet adhesiveness following stimuli causing mobilisation of body fat are described. The stimuli used were adrenocortical stimulating hormone (ACTH) in rabbits and in man, surgery, cigarette smoking, exercise, starvation, noradrenaline infusion and isoprenaline. The lipid and

platelet adhesiveness changes resulting from these stimuli are detailed and compared with the findings of other workers.

In Section III, the effects on platelet adhesiveness of the oral administration of substances known to affect lipid metabolism were investigated. These substances were fat, glucose, sucrose, γ -linolenic acid, and clofibrate. The platelet adhesiveness and serum lipid changes resulting from the administration of these substances are described and the relevant literature reviewed.

Section IV includes a review of the thesis along with the conclusions from the experimental work carried out.

The main conclusions concerning platelet adhesiveness are:-

- 1) Measurement by the Payling Wright method is technically simple and is reasonably reproducible.
- 2) Variation in the interval between venepuncture and measurement of platelet adhesiveness affects the result. The longer this interval is, the greater the adhesiveness will be. For measurements to be comparable this interval must be kept constant.
- 3) Variation in platelet adhesiveness from day to day is greater than that simply due to experimental errors.
- 4) Platelet adhesiveness is not influenced by the age or sex of the subject.
- 5) It is increased significantly in subjects with ischaemic heart disease.
- 6) It is not increased by the acute effects of cigarette smoking.
- 7) It is unaffected by sucrose in the diet.
- 8) It is not increased in obese subjects and is not affected by weight reduction.
- 9) It is increased by exercise.
- 10) It is increased by minor stress such as the embarrassment of smoking an unlit cigarette.
- 11) It is increased by major stress in the form of a surgical operation.
- 12) It is increased by noradrenaline and isoprenaline.

- 13) It is increased following the oral administration of fat.
- 14) It is also increased following glucose given orally.
- 15) It is not altered by the oral administration of γ -linolenic acid.
- 16) It is not altered by clofibrate.
- 17) Platelet adhesiveness is not influenced directly by changes in plasma NEFA.
- 18) It is not affected directly by changes in serum cholesterol.
- 19) It is not affected directly by changes in serum triglyceride.
- 20) Changes in blood sugar do not directly affect platelet adhesiveness.

No relationship could be demonstrated in vivo between platelet adhesiveness and serum lipids. The lipid and thrombogenic theories of atheroma production could therefore not be linked via platelet adhesiveness. The significance of the increased platelet adhesiveness found in subjects with ischaemic heart disease in relation to the development of atheroma is doubtful because of this, and because factors predisposing to ischaemic heart disease (such as cigarette smoking, obesity and possibly dietary sucrose) are not associated with increased platelet adhesiveness while protective factors (exercise and clofibrate) are not associated with decreased adhesiveness.

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SECTION I

INTRODUCTION AND METHODOLOGY

Chapter I

Introduction

In 1965, 23% of all deaths in Scotland were due to ischaemic heart disease (arteriosclerotic heart disease including coronary disease) and in the age group 50-55 years for men, this reached the high proportion of 41% (Annual Report of the Registrar General for Scotland, 1965). The problem of ischaemic heart disease appears to be particularly acute in Scotland, which along with South Africa (European population), Finland and the U.S.A., has one of the highest levels in the world (Office of Health Economics, 1966). The cause of this crippling and lethal disease is unknown.

The basic lesion is atheroma. There are two main theories concerning the formation of atheromatous plaques. Firstly there is what is usually termed the "Thrombogenic theory", which is associated with the names of Rokitansky (1852) and Duguid (1946). This theory states that the origin of the plaque is the deposition of thrombus on the intima of the blood vessel. This subsequently becomes incorporated into the intima, eventually resulting in the atheromatous plaque. The other "lipid theory" suggested by Virchow in 1862 and supported by Aschoff (1933) states that disordered lipid metabolism is the primary abnormality. Lipid derived by phagocytosis, by filtration from the blood, or by local synthesis, becomes deposited in the intima thus setting up a focus of irritation which results in plaque formation.

There are several pieces of evidence in support of each theory. Spontaneous thrombosis appears to occur, at least in animals, on the aorta (Geissinger, Mustard and Rowsell, 1962) and these thrombi consist mainly of platelets. Similar thrombi in extracorporeal shunts have a distribution around bifurcations and branches strikingly similar to the distribution of atheromatous plaques. (Murphy, Rowsell, Downie, Robinson and Mustard, 1962). Thrombosis however might not be spontaneous and may be initiated

by trauma to the vessel wall due to turbulence created around bifurcations and branches. It is interesting that Gutstein, Lazzarini-Robertson and La Taillade (1963) have shown that micro-trauma can result in the formation of an atheromatous plaque.

This experimental work in animals forms the basis of the thrombotic theory. In man, the evidence is less direct, although Duguid (1952) has shown that even large thrombi can become incorporated into the intima of vessels in man. Several indices of increased blood coagulability have been found in patients with ischaemic heart disease compared to controls. Thromboplastin generation, plasma fibrinogen and the Stypven clotting time fall into this category (McDonald & Edgill, 1957 & 1959). The most interesting abnormality however in the light of the animal experiments just quoted is increased platelet adhesiveness. This has been shown using different methods by McDonald & Edgill (1957) Owren, Hellem & Odegaard (1964), Nestel (1961) Besterman, Myat & Travadi (1967). Blood platelets could therefore be of central importance in the formation of the atheromatous plaque and in the accompanying thrombosis in ischaemic heart disease.

The problem of ischaemic heart disease is not directly related to the amount of atheroma however. For example in Jamaica (Robertson 1959) and Guatemala (Tejada & Gore 1957) atheroma is common while myocardial infarction is rare. Also Morris (1951) in a study of the necropsy records at the London Hospital found that although there had been a sevenfold increase in the number of cases of coronary heart disease, the amount of severe atheroma had in fact declined. If one accepts the idea that myocardial infarction is caused by coronary occlusion (and even this is disputed (Spain & Bradless 1960)), then, since the amount of atheroma does not parallel the amount of myocardial infarction, two thrombotic tendencies must be involved if the thrombogenic theory is correct, - one resulting in the formation of atheroma, the other in thrombosis over the plaque. This division into two separate components weakens this theory. It is further weakened by the fact that it does not explain the accumulation of lipid in the plaque although it has been shown that platelet cholesterol and phospholipid

levels appear to parallel the blood levels (Murphy, Rowsell, Downie, Robinson and Mustard 1962).

The lipid theory is supported by the presence of lipid in atheromatous plaques and by the ability of high cholesterol diets in rabbits to produce fatty deposits in the intima of large arteries. The presence of elevated blood lipids in patients who have had a myocardial infarction and also the increased incidence of myocardial infarction in those with elevated blood lipids - for example cholesterol (Dawber, Kannel, Revotskie and Kagan (1962) Morris, Kagan, Pattison, Gardner & Raffle (1966) strengthens this hypothesis. It has been said that the lipid theory does not account for thrombosis (Pickering 1964) but this is not entirely true since elevation of blood lipids by fat feeding can shorten the clotting time (Fullerton, Davie, Anastosopoulos 1953) increase platelet turnover (Mustard & Murphy 1962) and possibly inhibit fibrinolysis (Greig 1956, Gillman, Naidoo & Hathorn 1957 Billimoria, Drysdale, James & MacLaggan 1959) although this is disputed (Hougie & Ayers 1960 Ogston & Fullerton 1962). The lipid theory does not explain the distribution of plaques of atheroma within the arterial system however.

Whether the lipid or thrombogenic theory is accepted, the divergence of the incidence of myocardial infarction from the amount of atheroma suggests that at least two mechanisms are involved in the production of ischaemic heart disease. An alternative view would be that there are basically two types of atheromatous plaque, one which is essentially fibrous and the other consisting mainly of lipid which would in fact be an "atheromatous abscess". The latter type would tend to rupture and be complicated by thrombosis, while the fibrous plaque would be rather inert and be associated with a low incidence of ischaemic heart disease. This type of thinking still requires at least two independent processes producing the types of plaques at each end of the spectrum. Could the lipid and thrombogenic theories both be valid thus providing the two required independent processes? If this was the case then one would expect some common ground between them. The blood platelet which occupies a central position in the thrombogenic theory could be the point of focus between

the two theories since there is abundant evidence in the literature linking platelets and lipids in vitro. This evidence will be reviewed later in Chapter 6. The important point to be noted at this stage is that if this link could be substantiated in vivo, the platelet would form a bridge between the thrombogenic and lipid theories. The purpose of this thesis is to investigate the possibility of a link between plasma lipids and platelet adhesiveness, both of which are known to be abnormal in ischaemic heart disease.

Chapter 2

History and Review of Methods of Measuring Platelet Adhesiveness

Helen Payling Wright in 1941 was the first person to measure platelet adhesiveness quantitatively. Prior to this in 1927, Rowntree and Shionoya had observed platelet adhesion to the walls of extracorporeal shunts. They had attempted to measure this function of platelets by recording the time taken for the shunt to be completely obstructed. This proved to be very variable the circulation ceasing in 6 to 10 minutes usually "or at most in very large and vigorous animals in 25 minutes"! Best, Cowan & McLean in 1938 improved this technique by transilluminating a glass cell built into the shunt and was able to record the growth and formation of white thrombi. Payling Wright's method however eliminated the variation due to size and vigour of the animal by carrying out the measurement of platelet adhesiveness completely in vitro. Blood was withdrawn, heparinised and within 5 minutes was placed in a glass flask which was rotated at $3\frac{1}{2}$ r.p.m. This blood was sampled at regular intervals. Platelet counts were carried out on all the samples and were expressed as a percentage of the original count. She was able to show that platelets were adhering to the side of the glass flask. Since the area of glass with which the blood had been in contact was fixed for each sample, the number of platelets lost from the blood could be taken as a measure of their adhesiveness. Comparisons could therefore be made between the platelet adhesiveness of different people and of the same person at different times. Using this method she showed that the amount and type of anticoagulant added to the blood affected the platelet adhesiveness. Later, in 1942, she showed that platelet adhesiveness increased post operatively and like the platelet count was highest at the 10th day post operatively. Platelet adhesiveness appears then almost to have been forgotten until 1957 when interest was reawakened by the finding of Macdonald & Edgill (1957) that it was elevated in patients with ischaemic heart disease. Since then numerous publications have appeared on many aspects of this subject and inevitably the number of ways of measuring platelet adhesiveness has multiplied. These are summarised in Table I.

Table I

Methods of measuring platelet adhesiveness.

(a) In vitro

1. Adhesion of platelets to glass

- (a) Payling Wright (1941)
- (b) Hellem (1961)
- (c) Salzman (1963)
- (d) Moolton & Vroman (1949)
- (e) Caspary (1965 a)

2. Platelet to platelet adhesion

- (a) Born & Cross (1963)
- (b) Cunningham, McNicol & Douglas (1965)
- (c) Eastham (1964)
- (d) Hutcheson, Stark & Chapman (1959)

3. Platelet to platelet adhesion and platelet to glass adhesion

- (a) Stormorken, Lund-Rüse & Rorvik (1965)

(b) In vivo

- Borchgrevink (1960)

Several methods have been based on Payling Wright's in that they involve exposure of blood to a standard area of glass for a set time. Hellem's (1961) method involves the passage of citrated blood through a standard glass bead column. The time of contact between blood and glass is reduced to 23 sec. in this method compared to a minimum of 20 minutes using the Payling Wright method. This possible advantage is offset by the complexity of the apparatus required. The blood has to be pumped through the column at a standard rate and each column must be packed to the same density with glass beads of a standard size. A larger volume of blood is also required for this method. Hirsh, McBride & Wright (1966) have shown that there is a correlation between platelet adhesiveness measured by Hellem's method and that measured by Payling Wright's. Both methods have been shown to depend mainly on the release of adenosine diphosphate (ADP) from red blood cells (Gaarder, Jonsen, Laland, Hellem & Owren 1961; Caspary 1965 (b); Harrison & Mitchell 1966). The methods appear therefore to be measuring similar functions of platelets and to give similar results in spite of the marked difference in the time during which the blood is in contact with the glass. Salzman's modification (1963) of Hellem's method, on the other hand, in which the glass bead column is attached directly to the venepuncturing equipment and in which, therefore, platelet adhesiveness is measured prior to the addition of an anticoagulant, appears to measure a different platelet function. This is shown by the fact that when this method is made almost identical to that of Hellem's, by citration of the blood prior to its passage through the column, adhesiveness is abolished. (This apparent anomaly is explained by the increase in platelet adhesiveness with time after venepuncture (Hellem 1961) since the measurement is made by Salzman immediately after, compared to 15 minutes after venepuncture by Hellem). Moolton & Vroman (1949) passed blood through a glass wool filter but the surface area with which platelets are in contact is not standardised in this method. Caspary's method (1965 a) involves the adhesion of platelets to a glass slide of standard size which is agitated in blood in a polythene container. None of these methods which basically involve contact with glass appear to have any advantage over the original Payling Wright method.

There are several methods of measuring platelet to platelet adhesion. The best of these is that described by Born & Cross (1963). In this method, the optical density of platelet rich plasma in a transparent plastic container is measured by passing a light through the container onto a photo electric cell connected to a galvanometer. The optical density is kept uniform using a magnetic stirrer covered with plastic. This also causes the platelets to collide with one another. On addition of ADP or indeed any other agent causing platelet aggregation, the changes in optical density due to platelet clumping can be recorded against time, the greater the aggregation, the greater will be the increase in the transmission of light. This method has the advantage that the effects of various agents in differing concentrations can be compared. Platelet to platelet adhesiveness could therefore theoretically be determined by measuring the response to a standard amount of ADP. An objection to this is that some account would have to be taken of the initial platelet count in the platelet rich plasma. This could theoretically be overcome by expressing the change in optical density as a percentage of the difference in optical density between platelet rich and platelet poor plasma, since this correlates with the platelet count (Born & Cross 1963). Even when this is done however, Born and Cross have shown that the response to a standard dose of ADP diminishes with further centrifugation of platelets. This suggests that the more ADP sensitive or stickiest platelets tend to be preferentially centrifuged, an observation also made by Stormorken, Lund-Rüse & Rorvik (1965). This is a serious drawback which applies to all methods using platelet rich plasma. Changes in adhesiveness could be masked by variation in the number of platelets preferentially centrifuged.

Another measure of platelet to platelet adhesiveness is the time from addition of calcium to citrated or oxalated platelet rich plasma in a Chandler tube (1958) until the platelet snow storm appears prior to thrombus formation (Cunningham, McNicol & Douglas, 1965). Eastham (1964) measured platelet to platelet adhesiveness by adding ADP to heparinised blood in a polystyrene bottle which was rotated on a Matburn mixer for 30 minutes. EDTA was then added to stop aggregation and the platelet

count was compared with another sample which had been placed in EDTA originally. Prior to the development of this method, Hutcheson, Stark, & Chapman (1959) rotated platelet rich plasma at 33 r.p.m. on a turntable and measured platelet adhesiveness as the time taken for the platelet count to fall to 50% of the initial count. This unfortunately takes over 1 hour. These 3 methods basically measure platelet to platelet adhesiveness. Another method has been described by Stormorken, Lund-Rüse & Rorvik (1965) which appears to measure both platelet to platelet adhesiveness and platelet adhesiveness to glass. Platelet rich plasma after the addition of a standard amount of ADP solution is run through the standard glass bead column described by Hellem. The adhesiveness is expressed as described in Hellem's method. The object of this method is to eliminate the effect of packed cell volume which would be a variable when comparing adhesiveness between individuals. It is not clear however how this method compares with other methods. Certainly Rozenberg and Stormorken (1967) were unable to find any correlation between this method and Born & Cross's method measuring platelet to platelet adhesion only.

Borchgrevink in 1960 described a method of measuring platelet adhesiveness in vivo. A sphygmomanometer is inflated to 40 mm.Hg. on the upper arm and a cut 10mm x 1 mm deep made transversely on the volar surface of the forearm. The platelet count in the blood from the cut is used along with the count in venous blood to calculate the percentage of platelets adhering in the wound. This method would appear to suffer from several drawbacks. Values from different people will not be comparable since the diameter of arms is very variable and the blood flow to the arm with the cut will be variable as a result of this and other factors such as environmental temperature. The method also assumes even distribution of platelets in the peripheral circulation which is known not to be the case. The major difficulty however would appear to be the standardisation of the size of the cut.

After consideration of all the above methods of measuring platelet adhesiveness, the Payling Wright method was chosen for the following reasons:-

- 1) The equipment required is simple.
- 2) Measurement is simple
- 3) Only 2 ml. of blood are required.
- 4) The method is at least as good as other methods.
- 5) Platelet adhesiveness measured by this method is abnormal in subjects with ischaemic heart disease.

Chapter 3

Method and accuracy of platelet counting and adhesiveness measurements

The measurement of platelet adhesiveness by the Payling Wright method depends on the counting of platelets.

Platelet counting

There are many methods of counting platelets. The first examined was that used in the Haematology Department of the Western Infirmary. This method involved diluting one volume of blood in 20 volumes of freshly filtered 1% ammonium oxalate and counting the platelets under light microscopy. The ammonium oxalate caused the red cells to haemolyse and this in turn made the counting of platelets very difficult because red-cell remnants obscured the field. Also it was often difficult to distinguish between fragments of this debris, specks of dust and platelets. A basic requirement for a satisfactory method of platelet counting is confidence that one is actually counting platelets. This method did not meet this requirement.

The difficulty resulting from the red cell debris could be eliminated using a method which did not lyse red-cells. One such is the Rees Ecker method (1923) which has the further advantage of using brilliant cresyl blue, which stains the platelets. This produced the necessary degree of confidence, but the presence of red cells meant that a dilution of 1 in 100 was necessary in order that platelets were not missed when scanning the field. The dilution factor meant that several chambers had to be counted in order that sufficient platelets would be included.

Phase contrast microscopy was then tried. It was found to make little difference to the Rees Ecker method but when applied to the ammonium oxalate method, clearly overcame the difficulty of recognising platelets confidently. Platelets were easily identified by their bluish tinge, and this type of microscopy enabled one even to see pseudopodia extending from many of the platelets. The ammonium oxalate method using

phase contract microscopy was first described by Brecher & Cronkite in 1950. This method was finally adopted because only a dilution of around 1 in 20 was required and platelets could easily and confidently be identified and distinguished from dust and red cell debris.

Technique and Accuracy of Platelet Counting

All platelet counts were carried out on citrated blood. The technique of venepuncture and dilution of the blood with citrate will be described in the next section "Technique of Measuring Platelet Adhesiveness".

0.2 ml. of the citrated blood was diluted in 4 ml. freshly filtered 1% ammonium oxalate in a polystyrene container. In the case of the citrated blood, the pipette was washed out by sucking the diluting fluid in and out several times. The polystyrene container was then stoppered and the contents mixed gently. After several minutes, haemolysis of the red cells occurred and the platelet count could thereafter be carried out. A sample of this solution was placed in a Neubauer chamber and the platelets were allowed to settle for about 15 minutes. Evaporation was prevented during this time by having the chamber in a Petri dish containing moist cotton wool. The platelets were then counted using phase contrast microscopy. The area which was counted was variable. At least one column (0.2 sq.mm) of the central square on each half of the chamber was counted. If the total number of platelets in this area was less than 400, then further 0.2 sq.mm columns were counted until the total number of platelets counted was greater than 400. The number of platelets in 1 Cu.mm. was then evaluated by multiplying by 1050 and dividing by the number of 0.2 sq.mm. columns counted. The counts thus obtained were then corrected to the nearest 100. It should be emphasised that the counts quoted in this thesis are counts on citrated blood. 10% should be added to the quoted platelet count to obtain the count in whole blood.

The accuracy was checked by carrying out platelet counts on 2 occasions within 20 minutes on 31 subjects. The errors involved included the technique of venepuncture, dilution with anticoagulant, dilution with ammonium oxalate, and the error due to variation in the counting chambers.

Table II

Duplicate platelet counts (/cmm)

| Estimate I | Estimate 2 | Difference |
|-----------------|------------|------------|
| 209,000 | 193,600 | 15,400 |
| 154,600 | 155,400 | 800 |
| 188,400 | 180,400 | 8,000 |
| 208,000 | 218,000 | 10,000 |
| 242,800 | 208,800 | 34,000 |
| 259,200 | 265,200 | 6,000 |
| 257,400 | 228,600 | 28,800 |
| 165,200 | 167,000 | 1,800 |
| 131,500 | 134,500 | 3,000 |
| 165,000 | 173,500 | 8,500 |
| 168,600 | 167,800 | 800 |
| 177,000 | 169,400 | 7,600 |
| 190,000 | 199,000 | 9,000 |
| 136,000 | 134,000 | 2,000 |
| 218,700 | 217,000 | 1,700 |
| 109,200 | 112,000 | 2,800 |
| 121,800 | 118,600 | 3,200 |
| 186,000 | 193,000 | 7,000 |
| 129,300 | 124,300 | 5,000 |
| 158,000 | 143,400 | 14,600 |
| 167,500 | 165,500 | 2,000 |
| 113,500 | 104,000 | 9,500 |
| 161,000 | 153,000 | 8,000 |
| 179,000 | 181,000 | 2,000 |
| 219,000 | 196,000 | 23,000 |
| 160,500 | 133,000 | 27,500 |
| 261,600 | 238,000 | 23,600 |
| 144,000 | 142,200 | 1,800 |
| 138,800 | 143,800 | 5,000 |
| 149,000 | 148,200 | 800 |
| 100,400 | 100,600 | 200 |
| Mean Difference | | 8,830 |
| S.D. | | 9,270 |

The duplicate counts were counted at random among other platelet counts. The checking therefore included all the variables and each count is therefore a completely separate platelet count. The duplicate counts are shown in Table II. The coefficient of variation (V) was calculated from the formula $V = \frac{SD \times 100}{M}$ where $S.D. = \sqrt{\frac{\sum 2(x-m)^2}{N}}$ and in which S.D. is the standard deviation of the mean of duplicate platelet counts, M. is the mean of all platelet counts, X is one of the duplicate counts, m is the mean of duplicate counts and N is the number of duplicate counts. The coefficient of variation is 5.3%. This compares favourably with that found by Bennett (1967 b) of 5.7% and by Borchgrevink (1960) of 7.1%. Both of these workers assessed the coefficient of variation using the above formula for duplicate counts and both were using Brecher & Cronkite's method. The figures therefore are directly comparable.

Technique of measuring platelet adhesiveness

As already stated the method chosen was basically that of Payling Wright. Several modifications were made. Blood was withdrawn by clean venepuncture from a forearm vein, using a Gillette no.1 disposable needle and a polystyrene syringe. If the venepuncture was not clean or if air bubbled into the syringe, the syringe and needle were discarded and blood was withdrawn using fresh equipment from a different vein. A tourniquet was used to distend the vein but excessive stasis was avoided in view of the conflicting evidence of its effect on platelet adhesiveness (Sarajas & Myllyla 1964; Stormorken, Lund-Ruse, Rorvik 1965). After the blood had been drawn into the syringe, the needle was removed and 4.5 ml. of blood were mixed with 0.5 ml. of 3.8% sodium citrate in a polystyrene container. The blood and citrate were then gently mixed. 2 ml. of this mixture were then transferred at a fixed time after venepuncture using another polystyrene syringe into a 50 ml. spherical Quickfit glass flask of a standard diameter. This flask was rotated around its long axis for exactly 20 minutes at $3\frac{1}{2}$ r.p.m. Platelet counts were then carried out on the blood in the polystyrene container (initial count) and in the glass flask (final count). The difference between the counts gave the number of platelets which had adhered to a standard area of glass in 20 minutes. This number expressed

Table III

Duplicate measurement of platelet adhesiveness

| Estimation I | Estimation 2 | Differences between duplicates | Differences between Estimates 1 & 2 |
|------------------|--------------|--------------------------------|-------------------------------------|
| 15.9 | 14.7 | 1.2 | - 1.2 |
| 20.7 | 21.9 | 1.2 | + 1.2 |
| 28.1 | 24.1 | 4.0 | - 4.0 |
| 19.5 | 22.4 | 2.9 | + 2.9 |
| 21.6 | 24.8 | 3.2 | + 3.2 |
| 18.6 | 20.8 | 2.2 | + 2.2 |
| 23.8 | 13.8 | 10.0 | -10.0 |
| 32.8 | 28.0 | 4.8 | - 4.8 |
| 27.5 | 26.0 | 1.5 | - 1.5 |
| 19.7 | 29.7 | 10.0 | +10.0 |
| 27.3 | 28.1 | 0.8 | + 0.8 |
| 20.3 | 27.2 | 6.9 | + 6.9 |
| 16.3 | 22.1 | 5.8 | + 5.8 |
| 49.9 | 48.0 | 1.9 | - 1.9 |
| 49.7 | 56.2 | 6.5 | + 6.5 |
| 55.4 | 53.4 | 2.0 | - 2.0 |
| 47.0 | 50.1 | 3.1 | + 3.1 |
| 46.1 | 42.2 | 3.9 | - 3.9 |
| 25.1 | 17.7 | 7.4 | - 7.4 |
| 41.8 | 42.6 | 0.8 | + 0.8 |
| 36.7 | 32.7 | 4.0 | - 4.0 |
| 40.0 | 47.0 | 7.0 | + 7.0 |
| 47.2 | 43.7 | 3.5 | - 3.5 |
| 35.1 | 33.7 | 1.4 | - 1.4 |
| 38.8 | 51.0 | 12.2 | +12.2 |
| 48.3 | 53.4 | 5.1 | + 5.1 |
| 47.5 | 47.4 | 0.1 | - 0.1 |
| 38.9 | 47.2 | 8.3 | + 8.3 |
| 19.7 | 32.3 | 12.6 | +12.6. |
| 45.5 | 40.1 | 5.4 | - 5.4 |
| 47.8 | 52.3 | 4.5 | + 4.5 |
| Mean Differences | | 4.65 | + 1.4 |
| S.D. | | 3.4 | 5.6 |

as a percentage of the count of the blood in the polystyrene container (initial count) gave the platelet adhesiveness. Platelet adhesiveness therefore = $\frac{\text{initial count} - \text{final count}}{\text{initial count}} \times 100$.

Accuracy of Platelet adhesiveness measurement

The accuracy of measuring platelet adhesiveness was assessed in exactly the same way as in the case of the platelet count i.e. by carrying out 31 duplicate determinations. The results of these are shown in Table III.

It can be seen that duplicates can vary considerably. The mean difference (\pm S.D.) between duplicates was 4.65 ± 3.4 . This means that in order to be 95% certain that 2 measurements of platelet adhesiveness are different, there would have to be a difference of over 11%. Payling Wright did not assess the reproducibility of her method, but Millac (1967a) using a modification of the Payling Wright method similar to the present one, found the standard deviation of replicate determinations to be 4.4%. This means that 95% of his results would be within a range of 17.6% (4 times the standard deviation). Put another way, there were differences of at least 17.6% between some measurements of platelet adhesiveness on the same blood sample. Clearly the present results are better than this, since the greatest difference between duplicates was 12.6%.

The fact that fairly large differences between duplicates can occur, means that there is little point comparing two isolated platelet adhesiveness measurements. The investigations in this thesis, are designed mainly on the basis of repetition of the same experiment with analysis of the differences between platelet adhesiveness before and after a stimulus using Student's t test. For example, in the above experiment, it is clear there is no change in platelet adhesiveness between estimate 1 and estimate 2. This in a sense is a control experiment as nothing happened to the subjects between the two measurements. The difference of +1.4 (\pm SEM of 1.0) between the means is statistically insignificant. This result is similar to that obtained by Bennett (1967b) who found that the difference in platelet adhesiveness before and after a

drink of water (i.e. also a control type of experiment) was $-1.8^{+2.1}$ using Hellem's method.

This method of measuring platelet adhesiveness departs from the original description of the Payling Wright method in several ways.

- 1) Sodium citrate was used as an anticoagulant instead of heparin. When heparin was used platelet counting was difficult because platelet aggregation usually occurred. This was also the experience of Perkins Osborn and Gerbode (1958) who found that platelet aggregation could be prevented by small amounts of citrate. When sodium citrate was used there was no problem with platelet aggregation and platelet counting was easy.
- 2) There was no window in the side of the glass flask. This was used by Payling Wright to show that platelets were adhering to the glass and is unnecessary for measuring for platelet adhesiveness.
- 3) The time elapsing between venepuncture and measurement of platelet adhesiveness was standardised. The effect of varying this interval, when measuring platelet adhesiveness by the Payling Wright method, was unknown. This was therefore, investigated (Fyfe and Hamilton, 1967a) and will now be described.

Chapter 4

Effect of Variation of the interval between Venepuncture and measurement of platelet adhesiveness

Method

18 ml. of blood was withdrawn by clean venepuncture into a polystyrene syringe containing 2 ml. of 3.8% sodium citrate solution. The blood and citrate were gently mixed and transferred into a stoppered polystyrene tube. A stop-watch was started at the time of venepuncture, and platelet adhesiveness was measured, commencing 3, 5, 7 and 20 minutes after venepuncture in each of ten subjects and 5 and 20 minutes after venepuncture in a further twenty five subjects.

Results

The results for each of the ten subjects are shown in Table IV. The mean results with the standard errors of the means are shown in figure I.

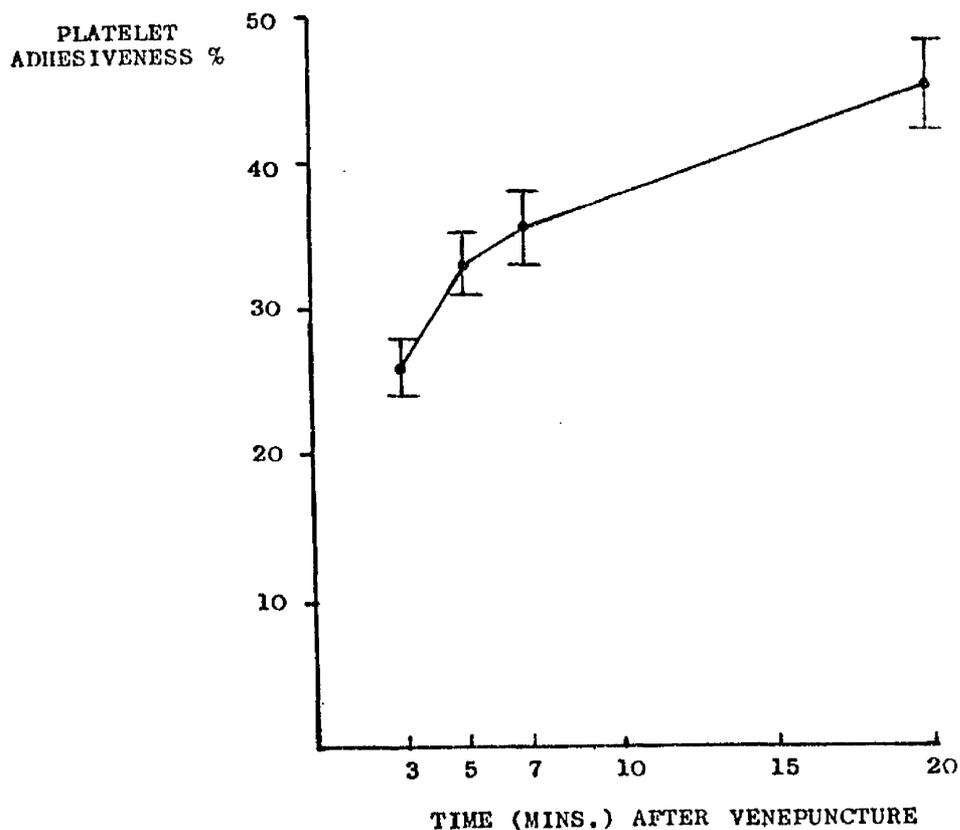


Figure I. Average platelet adhesiveness (%) \pm S.E.M in ten subjects at 3, 5, 7, and 20 minutes after venepuncture.

The values at 5, 7 and 20 minutes differed significantly from the 3 minute value ($p < 0.05$, < 0.01 , < 0.01 respectively). The mean values for platelet adhesiveness at 5 and 7 minutes did not differ significantly from each other, but both were significantly different from the 20 minute value ($p < 0.01$ and $p < 0.05$ respectively). The initial platelet count was not significantly altered.

Table V shows the mean results for platelet adhesiveness measured at 5 and 20 minutes after venepuncture in thirty five subjects, including the ten in Table IV. This larger series confirmed the increase in platelet adhesiveness with time after venepuncture and disclosed a significant correlation between platelet adhesiveness measured at 5 minutes and that measured at 20 minutes after venepuncture.

PLATELET ADHESIVENESS %
20 MIN. AFTER VENEPUNCTURE

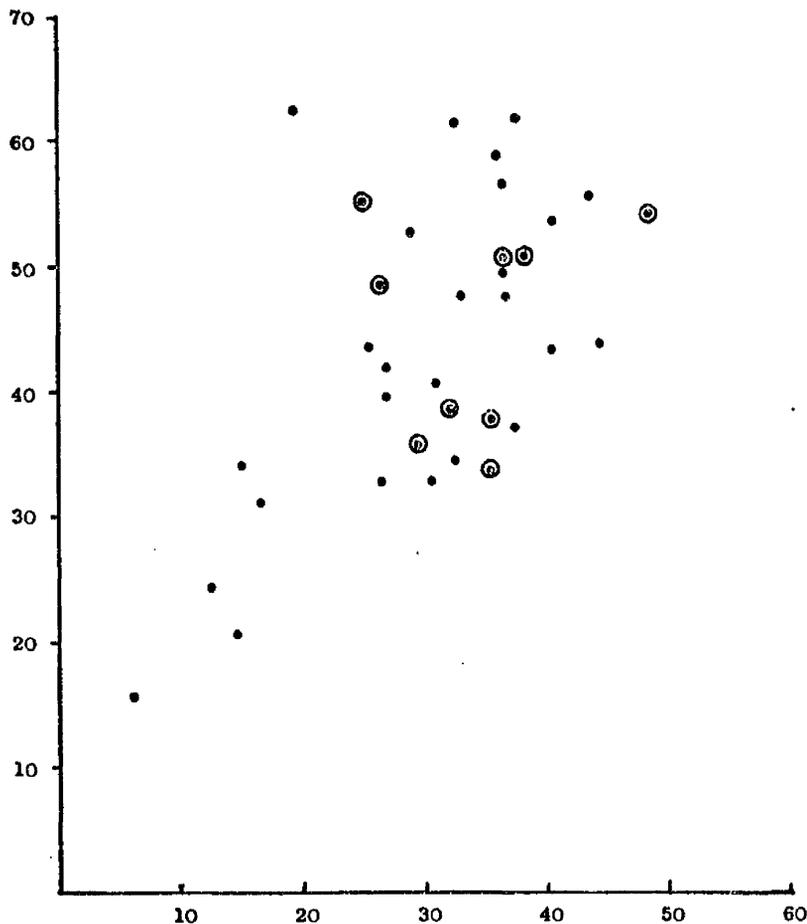


Figure 2.

Relationship between platelet adhesiveness measured five minutes after venepuncture with that measured 20 minutes after venepuncture in 35 subjects. Results for subjects with ischaemic heart disease are circled.

PLATELET ADHESIVENESS %, 5 MIN. AFTER VENEPUNCTURE

Table IV Platelet adhesiveness at 3, 5, 7 and 20 minutes after venepuncture in 10 subjects.

| Subject No. | Time after venepuncture (min.) | | | |
|-------------|--------------------------------|------|------|------|
| | 3 | 5 | 7 | 20 |
| 1 | 22.9 | 26.6 | 34.3 | 39.4 |
| 2 | 26.6 | 29.0 | 32.3 | 52.6 |
| 3 | 27.0 | 32.2 | 44.0 | 34.5 |
| 4 | 18.8 | 26.9 | 22.3 | 41.8 |
| 5 | 17.2 | 26.3 | 35.9 | 32.7 |
| 6 | 25.5 | 32.5 | 48.6 | 61.3 |
| 7 | 21.9 | 36.7 | 31.9 | 49.9 |
| 8 | 25.4 | 43.4 | 31.6 | 55.3 |
| 9 | 40.9 | 44.4 | 42.3 | 43.7 |
| 10 | 33.9 | 35.4 | 35.2 | 37.6 |
| Mean | 26.0 | 33.3 | 35.8 | 44.9 |
| + S.D. | +7.0 | +6.8 | +7.6 | +9.4 |

Table V Mean platelet adhesiveness in 35 subjects at 5 and 20 minutes after venepuncture.

| | Time after venepuncture (min.) | |
|---|--------------------------------|-----------------|
| | 5 | 20 |
| Platelet adhesiveness: mean \pm S.D.. | 30.7 \pm 9.6 | 43.6 \pm 11.9 |
| t Test | 5.0435 | P < 0.001 |
| Correlation coefficient... .. | r=0.5891 | P < 0.001 |

Discussion

Hirsh et al. (1966) reported a correlation between platelet adhesiveness measured by the method of Payling Wright and that measured by the method of Hellem. It is not surprising therefore that both methods should reveal an increase in platelet adhesiveness with time after venepuncture. The magnitude of the change in this investigation was similar to that found by Hellem.

As originally described, Payling Wright's method should be carried out within 5 minutes of venepuncture. Many published reports refer to the modification of McDonald and Edgill (1957) of the Payling Wright method, but the time of measurement in that paper was not stated and presumably was also within 5 minutes of venepuncture. To assess the results of investigations of platelet adhesiveness the timing of the measurement after venepuncture, and whether this was rigidly controlled, should be known. Since it was found that platelet adhesiveness measured 5 minutes after venepuncture and that measured 20 minutes after venepuncture are correlated, the timing of the measurement seems relatively unimportant, provided that it is constant. Errors arising from timing are less likely if the measurement is carried out, not in the first 5 minutes when platelet adhesiveness appears to be increasing rapidly, but later when the rate of increase is slower. This reasoning led Hellem to measure platelet adhesiveness 15 minutes after venepuncture. The time between venepuncture and measurement of platelet adhesiveness was therefore controlled accurately in all experiments in this thesis.

It was suggested subsequently by Holdrinet, Ewals & Haanen (1967) that the increasing adhesiveness may have been due to platelet aggregation and that this might have been caused by release of ADP from platelets during standing. We did not agree with this however since no aggregation was observed and because aggregation would have tended to lower the initial platelet count thereby reducing not increasing platelet adhesiveness (Fyfe and Hamilton 1967 b). Harrison & Mitchell (1966) and also Caspary (1965 b) have shown that platelet adhesiveness as measured by the Payling Wright method is mainly dependent on ADP released from red cells, not platelets.

It has been shown by Holdrinet et al. and also by Constantine (1965) that platelets become more responsive to ADP with time following venepuncture. A more likely explanation of the phenomenon would be that it is due to increasing sensitivity of platelets to ADP combined with increasing release of ADP from red cells.

Chapter 5

Variation in Platelet Adhesiveness with age, sex and diagnosis

The 5 minute adhesiveness results for the 35 subjects in whom platelet adhesiveness was measured at 5 minutes and at 20 minutes after venepuncture (Table V; figure 2) were analysed with respect to age, sex and diagnosis. The subjects had been chosen completely at random. There were 18 males and 17 females and the average age of the group was 47.7 years. There were 9 subjects (8 males and 1 female) with clinical or ECG evidence of ischaemic heart disease, the other diagnoses being:-

| | |
|-------------------------|---|
| asthma..... | 5 |
| normal..... | 4 |
| duodenal ulcer..... | 3 |
| hypothyroidism..... | 3 |
| thyrotoxicosis..... | 2 |
| rheumatoid arthritis... | 2 |
| diabetes..... | 2 |
| hypertension..... | 1 |
| albuminuria..... | 1 |
| renal failure..... | 1 |
| pneumothorax..... | 1 |
| vaso vagal attack..... | 1 |

Results

The average platelet adhesiveness for the whole group was 30.7%. A graph showing platelet adhesiveness against the age of the subjects is shown in figure 3. There was no correlation between the two, the correlation coefficient (r) being 0.101.

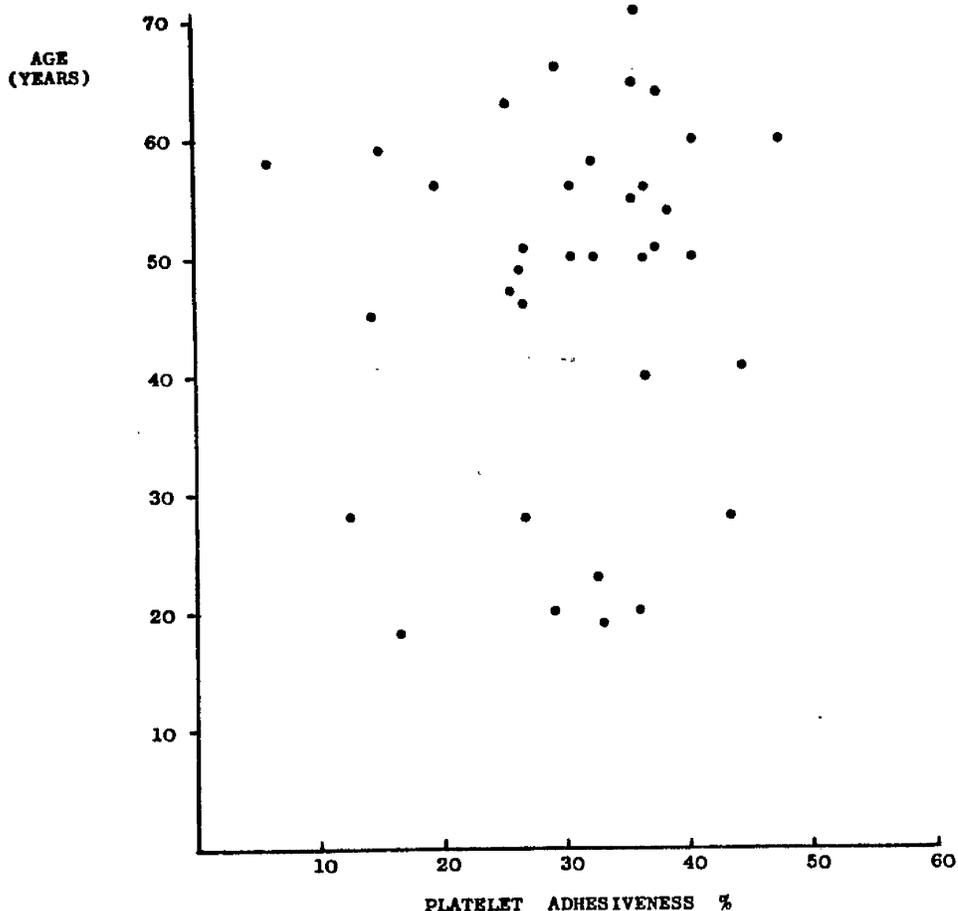


Figure 3. Graph of platelet adhesiveness against the age of each subject.

The mean platelet adhesiveness (\pm SD) for males was $31.4 \pm 8.4\%$ and for females was $30.0 \pm 11.0\%$.

The subjects with ischaemic heart disease had a higher mean platelet adhesiveness compared to the others ($34.2 \pm 7.1\%$ compared to 29.5 ± 10.2). This difference however, was not statistically significant. Platelet adhesiveness in subjects with ischaemic heart disease appeared to increase at the same rate as the 'controls' (see figure 2). This was confirmed arithmetically by dividing the 20 minute value by the 5 minute value for each subject.

Discussion

The method of measuring platelet adhesiveness is based on that of Payling Wright. Unfortunately, Payling Wright did not use citrate as anticoagulant and therefore one cannot compare the overall average of 30.7% with any of her results. All methods of measuring platelet adhesiveness adjust the area of glass in contact with the blood in a given time, to give adhesiveness values of this order. The combination of area of glass and time of contact appeared therefore to be satisfactory.

There was no correlation between platelet adhesiveness and the age of the subject, agreeing with the findings of Hirsh and McBride (1965) and of Millac (1967 b). The former workers did not find any difference in platelet adhesiveness between males and females and this too has been confirmed in the present study.

The average platelet adhesiveness for the subjects with ischaemic heart disease was higher than the others. The difference was not statistically significant because there was considerable overlap between the groups and because the number of subjects was relatively small.

Other workers have found considerable overlap between the platelet adhesiveness of subjects with ischaemic heart disease and controls (McDonald & Edgill 1957 and 1959; Owren, Hellem & Odegaard 1964; Nestel 1961 Besterman, Myat & Travadi 1967). The difference therefore requires fairly large numbers to demonstrate it. One could certainly not diagnose ischaemic heart disease by measuring platelet adhesiveness. It can be seen from figure 2 that there is considerable overlap between the subjects with ischaemic heart disease and other subjects. If platelets are involved in the formation of atheroma and platelet adhesiveness has any relevance to the problem, then this could be due to subjects having extensive atheroma but no clinical evidence of it. If this were true, one might expect some increase in platelet adhesiveness with age, but this was not found. What ever the reason platelet adhesiveness is not a good discriminator of ischaemic heart disease but is generally agreed by the

workers already named to be elevated in ischaemic heart disease.

Several groups of workers have denied that platelet adhesiveness is elevated in ischaemic heart disease. Using the Payling Wright method, Weiner, Zeltmacher, Reich & Shapiro (1948) found that the platelet adhesiveness in 5 subjects with arteriosclerotic heart disease was within normal limits. Clearly this denial is not well founded. It has to be contrasted with the significant difference demonstrated by McDonald & Edgill (1957) when comparing 48 subjects with ischaemic heart disease with 48 age and sex matched controls. Gooding & Enticknap (1967) using Hellem's method mention that they did not find increased adhesiveness in subjects with ischaemic heart disease. They have not yet presented the evidence for this however. Eisen, Tyson, Michael & Baumann (1951) and Bobek & Cepelak (1958) both using the Moolten and Vroman glass wool filter method, have suggested that platelet adhesiveness is normal in ischaemic heart disease. These workers' requirements for abnormality have been values above the normal range. As already stated there is considerable overlap between normality and ischaemic heart disease in regard to platelet adhesiveness.

The subjects with ischaemic heart disease did not have a greater than normal increase in platelet adhesiveness with time after venepuncture. This means that any increased adhesiveness demonstrated in vitro is likely to be present also in vivo. If the increase had been greater than normal then the in vitro adhesiveness could have been increased while the in vivo adhesiveness of platelets could have been normal.

One might suggest that packed cell volume differences could explain the differences in platelet adhesiveness between subjects with ischaemic heart disease and controls since as already mentioned ADP from red cells is the main factor influencing the measurement. There is no way of standardising the packed cell volume since some form of centrifugation would be required. This could result in delay prior to the measurement of adhesiveness being carried out and also the problem of preferential centrifugation of 'sticky' platelets would arise. McClure, Ingram, Stacey, Glass & Matchett (1966) corrected for packed cell volume

by assuming that the regression is linear and that the regression coefficient of platelet adhesiveness on haematocrit was unity. They therefore standardised all platelet adhesiveness measurements to correspond to a haematocrit of 43%. If therefore platelet adhesiveness proved to be 40% in patient with a haematocrit of 53% then the platelet adhesiveness would be corrected by 10% to 30%. This on the admission of these authors is a gross over-simplification. In the present thesis, this problem was overcome by investigating alterations in adhesiveness under various conditions rather than the absolute values. Minor alterations of the haematocrit are unlikely to have any significant effect on platelet adhesiveness and therefore no correction was made.

Chapter 6

Evidence linking platelets with blood lipids

1) Cholesterol

Macdonald & Edgill in 1957 showed that subjects with ischaemic heart disease had significant elevation of platelet adhesiveness compared to controls. They subsequently demonstrated that this elevation of platelet adhesiveness could be lowered by putting the subjects on a rice and fruit diet. (McDonald & Edgill 1958). This diet also lowered their serum cholesterol and this suggested that there might be a relationship between the two since both were lowered by the diet. Lowering of the serum cholesterol by another measure, clofibrate therapy, was also found to lower platelet adhesiveness (Symons, deToszeghi & Cook (1964); Carson, McDonald, Pickard, Pilkington, Davies & Love (1966) but recently this has been challenged by O'Brien & Heywood (1966). The fact that subjects with ischaemic heart disease have elevation both of their serum cholesterol and of platelet adhesiveness and that both can be lowered together by one measure is a basis of a possible association between the two.

2) Triglyceride

A similar argument can be used to link serum triglyceride with platelet adhesiveness since serum triglyceride levels have been shown to be elevated in subjects with ischaemic heart disease (Albrink, Meigs & Man 1961) and they too can be lowered by clofibrate therapy (Oliver, 1963, Hillman, Zumoff, Kessler, Kara, Rubin & Rosenfeld 1963).

Philip and Payling Wright (1965) showed that 1½-2 hours after a fatty meal platelet adhesiveness was increased compared to fasting values. They showed that the optical density of the plasma was also increased at this time and therefore suggested that the lipaemia may have been the factor responsible for the increase in platelet adhesiveness. They did not analyse this further, merely speculating that it could either have been the elevation of the serum triglyceride (which was being measured by the

optical density method) or of non-esterified fatty acids which was responsible. Further investigation into this post-prandial elevation of platelet adhesiveness will be described later.

Serum triglyceride has been measured as part of the present investigations to determine whether there might be an association between serum triglyceride and platelet adhesiveness.

3) Non-esterified fatty acids

The bulk of the evidence suggesting a relationship between platelets and lipids incriminates non-esterified fatty acids. This relationship will be discussed under the following headings:-

- a) Relationship between fatty acids and vascular occlusion.
- b) Fatty acids and the coagulation mechanism.
- c) Effects of fatty acids on platelets in vitro.
- d) The effect of albumin on the interaction between fatty acids and platelets.
- e) Effects of fatty acids on platelets in vivo in animals.
- f) Evidence linking fatty acids and platelets in man.
- g) Summary and conclusions.
- h) Lines of research suggested by this evidence.

The formulae of the fatty acids mentioned subsequently in the text are shown in Table VI.

a) Relationships between fatty acids and vascular occlusion

Injections of long chain saturated fatty acids into dogs are capable of producing widespread thrombosis and death (Connor, Hoak & Warner, 1963; Hoak, Connor, Eckstein & Warner, 1964). When injected into the artery of the wing of a bat they can produce thrombosis with marked vasoconstriction while unsaturated fatty acids (oleic (18/1) and linoleic (18/3)) acids do not have this effect (Soloff & Weidman 1963). These experiments were carried out with the sodium salts of the fatty acids in aqueous suspending media, the fatty acids existing as colloidal particles known as micelles. In vivo, non-esterified fatty acids are normally bound to albumin (Goodman 1958 a) and therefore these experiments are

Table VI. Formulae of fatty acids and related substances mentioned in text.

| | | |
|-------------------|-------------------------|--|
| ^C 22:0 | behenic acid | $\text{CH}_3(\text{CH}_2)_{20}\text{COOH}$ |
| ^C 20:0 | arachidic acid | $\text{CH}_3(\text{CH}_2)_{18}\text{COOH}$ |
| ^C 18:0 | stearic acid | $\text{CH}_3(\text{CH}_2)_{16}\text{COOH}$ |
| ^C 18:1 | oleic | $\text{CH}(\text{CH}_2)_7\text{COOH}$ 11 $\text{CH}(\text{CH}_2)_7\text{CH}_3$ |
| ^C 18:1 | ricinoleic acid | $\text{CHCH}_2\text{CH}(\text{OH})(\text{CH}_2)_5\text{CH}_3$ 11 $\text{CH}(\text{CH}_2)_7\text{COOH}$ |
| ^C 18:1 | elaidic acid | $\text{CH}_3(\text{CH}_2)_7\text{CH}$ 11 $\text{CH}(\text{CH}_2)_7\text{COOH}$ |
| ^C 18:2 | linoleic acid | $\text{CH}_3(\text{CH}_2)_4\text{CH}$ 11 CHCH_2CH 11 $\text{CH}(\text{CH}_2)_7\text{COOH}$ |
| ^C 18:3 | linolenic acid | $\text{CHCH}_2\text{CH}=\text{CHCH}_2\text{CH}_3$ 11 $\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$ |
| ^C 18:3 | γ linolenic acid | $\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_4\text{CH}_3$ 11 $\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_4\text{COOH}$ |
| ^C 16:0 | palmitic acid | $\text{CH}_3(\text{CH}_2)_{14}\text{COOH}$ |

Table VI (Continued) Formulae of fatty acids and related substances mentioned in text.

| | | |
|------------------------------|------------------|---|
| ^C 16:1 | palmitoleic acid | $\text{CH}_3(\text{CH}_2)_5 \text{CH} = \text{CH}(\text{CH}_2)_7 \text{COOH}$ |
| ^C 14:0 | myristic acid | $\text{CH}_3(\text{CH}_2)_{12} \text{COOH}$ |
| ^C 12:0 | lauric acid | $\text{CH}_3(\text{CH}_2)_{10} \text{COOH}$ |
| Prostaglandin E ₁ | | $\text{CH}_3(\text{CH}_2)_4 \text{CH}(\text{OH})\text{CH}=\text{CH}-\overset{\text{1}}{\text{CH}}-\overset{\text{1}}{\text{CH}}-(\text{CH}_2)_6 \text{COOH}$ $\begin{array}{c} \text{CH}(\text{OH}) \quad \text{C} = \text{O} \\ \quad \quad \quad \backslash \quad / \\ \quad \quad \quad \text{CH}_2 \end{array}$ |
| Clofibrate | | $\begin{array}{c} \text{CH}_3 \\ \text{1} \\ \text{Cl} \text{---} \text{C}_6\text{H}_4 \text{---} \text{O} \text{---} \text{C} \text{---} \text{COOC}_2\text{H}_5 \\ \text{1} \\ \text{CH}_3 \end{array}$ |

unphysiological. The addition of albumin to the fatty acids prior to their infusion diminishes their effect (Hoak, Connor, Eckstein & Warner 1964) but Hoak, Connor & Warner (1966) have found evidence of thrombosis following the injection of 5% albumin solution containing fatty acids in a concentration of 2,000 μ Eq./l. into rabbits while a control solution containing 200-400 μ Eq./l. fatty acid had no effect. The level of 2,000 μ Eq./l., they found could easily be exceeded in rabbits by mobilising fat by means of ACTH injections - a situation also complicated by thrombosis (Hoak, Poole & Robinson 1963). Histology of the thrombi resulting from fatty acid infusion showed that they were made up mainly of platelets (Hoak 1964) just as one would expect after thrombosis as distinct from clotting.

(b) Fatty acids and the coagulation mechanism

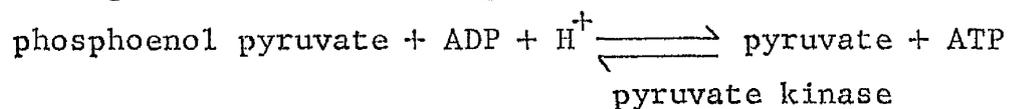
Fatty acids accelerate the coagulation mechanism. Poole (1955 a) found that sodium stearate, palmitate and oleate shortened the calcium clotting time while elaidate, linoleate and ricinoleate had no measurable effect. Sodium stearate also increased and shortened the time required for thrombin generation. Connor (1962) found that all saturated fatty acids accelerated thrombin formation in a Chandler tube while unsaturated fatty acids had no effect. The effect of saturated fatty acids increased with increasing chain length. Glass, Kaolin, Bentonite, which are known to activate Hageman factor, had an accelerating effect similar to the longest chain fatty acids (behenic (22/0) and arachidic (20/0)). The fatty acids did not accelerate thrombus formation of the blood of Peking ducks which are deficient in Hageman factor. For these reasons Connor believed that fatty acids activate Hageman factor. This was confirmed by experiments using Hageman factor deficient blood by Margolis (1962) and Didesheim & Mibashan (1963) but denied by Botti & Ratnoff (1963) who believed that they activated factor XI (P.T.A.).

(c) Effects of fatty acids on platelets in vitro

That fatty acids are able to aggregate platelets has been shown in

vitro by several groups of workers using basically the Born method. Generally the ability to aggregate platelets appears to depend on the saturation and chain length of the fatty acid. Mahadevan, Singh & Lundberg (1966) found that all saturated fatty acids were capable of inducing platelet aggregation and that the longer the chain, the greater was the aggregation produced. Of the unsaturated fatty acids tested, although in general they produced less aggregation, oleic acid was almost as potent as stearic acid. Linoleic and linolenic acids were ineffective, and linoleic acid appeared to protect platelets to some extent from the effect of behenic acid. Hoak, Warner & Connor (1967) however, found that stearic, oleic, linoleic and linolenic acids all produced platelet aggregation, stearic being the most potent. The aggregation produced by all the fatty acids appeared to be irreversible. The aggregating ability (of stearic acid) was markedly reduced in the plasma of the patient with Hageman factor deficiency. This could mean that the fatty acids were producing aggregation by Hageman factor activation. This was also suggested by Shore & Alpers (1963) who measured the effect of fatty acids on platelets by determining the amount of histamine released. Using this method they also found that the amount of histamine released depended on the chain length of the fatty acid. The reason for their suggesting that Hageman factor activation may have been a factor, was that when the platelets were suspended in buffered bovine albumin and therefore were not in contact with coagulation factors, there was no measurable platelet damage. Another interpretation of this however would be that the albumin solution had bound the fatty acids thereby protecting the platelets from their effect. Kerr, Pirrie, MacAulay and Bronte-Stewart (1965) using fatty acid lecithin sols found that of the fatty acids they tested all aggregated platelets apart from linoleate and linolenate. Linolenate provided a protective effect against aggregation by other fatty acids. The effects, as found by others, were dependent on chain length and saturation of the fatty acid. Ardlie, Kinlough, Glew & Schwartz (1966) using aqueous suspensions of fatty acids at concentrations much lower than used by other workers (approx. 1/10th of those of Kerr, Pirrie, MacAulay & Bronte-Stewart 1965) found that

palmitic, stearic, oleic, linoleic and linolenic acids did not produce platelet aggregation but that stearic acid caused enhanced clumping of platelets by ADP. Haslam (1964), suggested that the aggregation of platelets by fatty acids is mediated via ADP. This was suggested on the basis that both adenosine and pyruvic kinase with phosphoenolpyruvate could prevent platelet aggregation by fatty acids since pyruvic kinase with phosphoenolpyruvate "mops up" ADP in a solution by forcing the following reaction to the right:-



(d) The effect of albumin on the interaction between fatty acids and platelets

The obvious objection to all of these studies is that the fatty acids infused or used to aggregate the platelets were not in the "in vivo" state bound to albumin. Indeed incubation of stearic acid with albumin prior to its infusion into dogs prevented the expected thrombosis and death (Hoak, Connor, Eckstein & Warner 1964). In vitro platelet aggregation by fatty acids could also be markedly reduced by addition of albumin to the fatty acids (Hoak, Warner & Connor 1967). It would therefore appear that it is the binding of fatty acids to albumin which prevents disaster in the physiological situation. Goodman (1958 a) has investigated the binding of fatty acids to albumin. He found that there were 3 classes of binding site. The first class (tightest binding) consisted of 2 sites, the second of 5 and the third of approximately 20. Goodman (1958 b) subsequently showed that the second class binding sites on albumin had approximately the same association constant as binding sites on red blood cells. As red cell ghosts had a similar association constant he concluded that this binding of fatty acids to cell walls was independent of metabolic activity. He suggested that the avidity of cell membranes for fatty acids equal to that of second class albumin binding sites might be an important factor in explaining the very rapid transfer of fatty acids from blood to cells. If this is a generalised function of cell membranes then presumably

platelets would be affected also. Assuming the plasma albumin concentration to be 4G/100 ml., the molecular weight of albumin to be 66,000 and the number of tight binding sites on each albumin molecule to be 2, then saturation of the tight binding sites would occur at a plasma fatty acid concentration of 1212MEq/l. If fatty acids have a significant in vivo effect on platelets one would expect them to have a dramatic effect above this plasma concentration since the second class albumin binding sites would be in equilibrium with binding sites on cell walls. If these sites are important in the rapid transfer of fatty acids, then one would also expect some effect on platelets below these levels if the turnover of fatty acids were to be increased. On theoretical grounds there is therefore plenty of in vitro evidence to suggest that fatty acids might have important effects on platelets in vivo.

(e) Effects of fatty acids on platelets in vivo in animals

Small doses of fatty acids given I.V. to rabbits have been shown to produce thrombocytopenia lasting for 1-2 hours (Zbinden 1964). The most active acids were found to be myristic & lauric; activity diminished with increasing and decreasing chain length, although behenic acid had activity similar to lauric acid. The effect of unsaturated acids linoleic and linolenic was comparable to that of palmitic while oleic was only active at very high concentrations. Zbinden thought his results might indicate a direct effect of fatty acids on platelets because the relative activity of the various acids was quite different from the in vitro results of Margolis (1962) who had shown Hageman factor activation. (Margolis had found that the long chain saturated acids were most active while lauric and myristic acids were almost inactive). A more important point suggesting that the fatty acids had produced platelet aggregation rather than thrombosis was the return of the platelet count to normal levels in 2 hours. Intravascular haemolysis probably occurred in some cases as several rabbits had haemoglobinuria. This raised the possibility that ADP released from red cells could have produced the thrombocytopenia but as haemoglobinuria occurred in the case of injection of acids which did not produce

thrombocytopenia a direct effect on platelets would appear more likely.

(f) Evidence linking fatty acids and platelets in man

Turning to the evidence in man, it is interesting that thrombosis is said to be common in diabetics losing weight rapidly (Beckett & Lewis 1960). Diabetics are known to have increased platelet adhesiveness (Bridges, Dalby, Millar and Weaver 1965) and high plasma NEFA levels which would be expected to be even higher during a period of fat mobilisation. It is therefore tempting to incriminate plasma NEFA in the increased incidence of thrombosis. The elevation of platelet adhesiveness in ischaemic heart disease and in disseminated sclerosis - conditions in which fatty acid abnormalities have been claimed (James, Lovelock, Webb & Trotter, (1957), Baker, Thompson & Zilkha (1964) is also in favour of a link between fatty acids and platelets. It is interesting also that substances with a fatty-acid-like structure have been shown to affect platelet adhesiveness. Clofibrate, and prostaglandin E₁ whose formulae are shown in Table VI have a structural similarity to fatty acids and have been shown to affect platelets (Symons, de Toszeghi & Cook 1964; Carson, McDonald, Pickard, Pilkington, Davies and Love, 1963 and 1966; van Creveld and Pascha 1968). The ability of clofibrate to lower platelet adhesiveness has however been questioned (Millac, Caspary & Prineas, 1967 O'Brien & Heywood, 1966). Another interesting property of prostaglandin E₁ is its ability to inhibit the release of fatty acids from depot fat (Kupiecki, 1967; Nakano, Ishii & Gin, 1968). The effect of these substances on platelet adhesiveness therefore could involve a mechanism involving fatty acid metabolism.

When work for this thesis started a further piece of evidence favouring a link between fatty acids and platelet adhesiveness was the report by Owren, Hellem and Odegaard (1964) that linolenic acid was capable of reducing the elevated platelet adhesiveness found in ischaemic heart disease. This has since been denied by this group (Owren, Hellem & Odegaard 1965) and by others (Borchegrevink, Berg, Shaga, Skjaeggstad & Stormorken 1965).

(g) Summary and conclusions on relationship between fatty acids and platelets

The conclusion reached from all these studies would therefore appear to be that saturated fatty acids are capable of producing platelet aggregation. This effect appears to increase with increasing chain length. The effect of unsaturated fatty acids is less clear. On balance, they would appear to aggregate platelets although their effect is much less than that of saturated fatty acids. Kerr, Pirrie, MacAulay & Bronte-Stewart (1965) and Mahadevan, Singh & Lundberg (1966) suggest that linolenic acid may protect platelets from the aggregating effect of other fatty acids. It is interesting that these observations on the effect of linolenic acid would have been in accord with the now retracted (Owren, Hellem & Odegaard, 1965) and disproved (Borchgrevink et al. 1965) work of Owren, Hellem & Odegaard, (1964) which showed that platelet adhesiveness to glass appeared to be reduced by the administration of linolenic acid orally to subjects with ischaemic heart disease. The protective effect of linolenic acid on platelet aggregation in vitro has also been denied however by Hoak, Warner & Connor (1967). Hageman factor appears to be involved in the acceleration of the coagulation mechanism and may be involved in platelet aggregation by fatty acids. ADP is involved and is probably "the final common pathway" in the production of platelet aggregation by various substances. The effect of fatty acids on platelets appears to be prevented by the binding of fatty acids to albumin but this binding mechanism may become saturated at plasma FFA levels which can be obtained in certain physiological and pathological conditions.

(h) Lines of research suggested by this evidence

The question to be answered therefore is do endogenous fatty acids have any effect on platelet function in vivo? Platelet adhesiveness has been used as the indicator of an effect on platelets because fatty acids appear to aggregate platelets via the ADP mechanism or if present in much smaller amounts make platelets more sensitive to the aggregating effect of ADP. It has been suggested that fatty acids may cause a haemolytic anaemic and therefore another possibility would be that the fatty acids could act on platelets via the red cells by releasing ADP from them. Platelet

adhesiveness depends not only on the release of ADP from red cells but also on the sensitivity of platelets to ADP. If either of these mechanisms is in operation in vivo then one would expect to detect it by measuring platelet adhesiveness. A further reason for using platelet adhesiveness as the measure of platelet function is that it is known to be increased in subjects with ischaemic heart disease. If increased platelet adhesiveness is involved in the development of atheroma then this measure of platelet function would be the relevant one.

In the following experimental section of this thesis various stimuli have been used to mobilise endogenous fatty acids and the effect of this on platelet adhesiveness has been studied. In Section III, the effects on platelet adhesiveness of oral administration of substances known to affect fat metabolism have also been studied.

SECTION II

STUDIES OF PLATELET ADHESIVENESS FOLLOWING
STIMULI CAUSING MOBILISATION OF BODY FAT.

Chapter 7

Mobilisation of fatty acids in rabbits using adrenocortical stimulating hormone (ACTH)

Fatty acid infusions, as explained already, were thought to be unstable and completely unphysiological and were therefore not employed in this investigation. The first approach to the problem of the relationship between platelet adhesiveness and fatty acids was to induce fatty acid mobilisation by ACTH in rabbits in an experiment similar to that described by Hoak, Poole & Robinson (1963). These workers found that thrombosis and death was the usual outcome when large doses of ACTH (50 units/Kg.) were injected producing a five-to-seven-fold rise in plasma NEFA. They noticed a fall in the platelet count in 2 out of 5 rabbits 2 hours after ACTH but did not investigate this further. A similar experiment was designed for the present investigation.

Method and Results

Twelve rabbits were investigated. Basal blood samples were obtained by arterial puncture from the main artery supplying the ear. The rabbits were then given porcine ACTH subcutaneously. Four were given 50 units ACTH/Kg. and a further arterial sample was obtained after 2 hours. Four were given 25 units ACTH/Kg., the other 4 were given 10 units ACTH/Kg., and the post-ACTH specimens of blood were obtained after 1 hour in these two groups. The lower doses of ACTH were used in an attempt to reduce the mortality and to avoid in vivo platelet aggregation. The platelet count, platelet adhesiveness, packed cell volume, plasma NEFA and blood sugar were measured in all blood samples. Packed cell volume was measured using a Wintrobe tube after centrifuging at 3,000 r.p.m. for 30 minutes in a swing out centrifuge of 15. c.m. radius; Plasma NEFA by the method of Dole & Meinertz (1960) using Nile Blue as an indicator, the fatty acids being titrated with sodium hydroxide against palmitic acid standards in heptane; blood sugar using a Technicon Autoanalyser (Method N-2h) which measures total reducing substances.

In the high-dosage group, 2 of the 4 rabbits died within 24 hours and one of the others was killed. In the 25 units/Kg. group, one rabbit died. There were no deaths in the low-dosage group. Post-mortem examinations were carried out but failed to show any cause for the deaths. In particular, there was no evidence of thrombosis.

The results of the measurements made on the blood samples are shown in Table VII.

ACTH experiments

Table VII

| | Mean Values \pm S.D | | P value |
|---|-----------------------|------------------|---------|
| | Pre ACTH | Post ACTH | |
| platelet count $\times 10^3/\text{cmm}$ | 319 \pm 59.1 | 214 \pm 79.5 | < 0.001 |
| platelet adhesiveness % | 24.8 \pm 12.9 | 31.4 \pm 9.2 | N.S. |
| Non Adhesive Platelet Count $10^3/\text{cmm}$ | 235.3 \pm 51.9 | 141.0 \pm 43.3 | < 0.001 |
| P.C.V. % | 35.5 \pm 3.5 | 33.8 \pm 3.6 | < 0.01 |
| plasma NEFA Meq/l. | 166 \pm 82 | 1139 \pm 274 | < 0.001 |
| Blood glucose mg/100ml. | 68.3 \pm 23.1 | 93.1 \pm 29.7 | < 0.01 |

The platelet count fell in all cases. The PCV also fell in all but 2. The fall in the platelet count was greater than could be accounted for by the fall in the PCV and there was no correlation between the two. It was thought that the drop in the platelet count indicated intravascular aggregation of the platelets. One of the rabbits had an ear chamber and this allowed microscopy of the capillary circulation. After ACTH, platelet aggregates were seen within the circulation of this rabbit. Because of the fall in the platelet count after ACTH, the platelet adhesiveness estimations are not immediately relevant to the present studies, since if platelet adhesiveness is of any significance at all, the most 'sticky' platelets would be expected to aggregate first. This difficulty can be overcome by comparing the non-adhesive platelet counts before and after ACTH. It can be seen that there was a highly significant fall in the number of non-adhesive platelets, suggesting a true increase in platelet adhesiveness.

ACTH produced a dramatic rise in the plasma NEFA levels together with an increase in the blood glucose. The percentage changes in the NEFA and blood glucose correlated well ($r = 0.716; p < 0.01$). The percentage change in NEFA was significantly less after 10 units ACTH/Kg. than that after 25 units/Kg. The changes after 50 units/Kg. and after 25 units/Kg. were similar, probably due to the different timing of the post ACTH samples.

The percentage changes in blood glucose and plasma NEFA did not correlate with the percentage changes in platelet count or non-adhesive platelet count.

ACTH also produced a change in the appearance of the red cells. There appeared to be spicules around the red cell membrane, similar to "acanthocytosis" in man. This change was seen when counting the platelets in the Neubauer chamber using Rees Ecker diluting fluid. The red cells in the pre-ACTH specimen were always normal.

Since the changes in platelet adhesiveness were being masked by platelet aggregation in spite of reduction in the dose of ACTH, it was decided to try to prevent platelet aggregation using heparin and carry out the same experiment.

Effect of ACTH on platelets in the present of heparin

Six rabbits were investigated. A basal blood sample was withdrawn by arterial puncture from the main artery supplying the ear and following this, 500 units of heparin were injected intra-arterially. Each rabbit was then given 10 units ACTH/Kg. subcutaneously and a further arterial blood sample was obtained 1 hour later.

The results are shown in Table VIII. These results were compared with a control group which was treated in exactly the same manner except that no heparin was given. This group consisted of 7 rabbits, 4 of which had been included in the previous investigation in the 10 units ACTH/Kg. group.

It can be seen that heparin failed to prevent the drop in the platelet count following ACTH although this appeared to be less than in the non-heparin group (Table IX). The average percentage change in the heparin group was 11.05 ± 9.9 compared to 22.1 ± 20.4 in the non heparin group. However, this difference was not statistically significant. The fall in the non-adhesive platelet count was also greater in the control group but this difference also was not statistically significant.

Discussion

ACTH was shown by Di Girolamo, Rudman, Reid and Seidman (1961) to be capable of producing a tenfold rise in plasma NEFA in rabbits. The sevenfold rise found in the present series of experiments is of the same order and also similar to that found by Hoak, Poole and Robinson (1963). The in vitro work of White and Engel (1958) suggests that ACTH produces fatty acid mobilisation by acting directly on adipose tissue. The active principle may not in fact be ACTH but 'lipotropin' (Li, 1964) which is usually extracted along with it. The correlation between the change in blood sugar and the change in plasma NEFA suggests that the stimulus to both may be the same. Since adrenaline can produce both a rise in plasma NEFA and in blood sugar and since the rabbits were outwardly obviously in a stressed state as a result of the injection, adrenaline release may have been involved in the metabolic changes. ACTH is not thought to affect platelet adhesiveness (Millac 1967 c) but adrenaline is thought to

| | | Mean values \pm S.D. | | |
|--|---------|------------------------|-------------------|--------|
| | | Pre ACTH | Post ACTH | |
| platelet count x 10 ³ /cmm | heparin | 383.4 \pm 123.3 | 334.2 \pm 88.0 | < 0.05 |
| | control | 339.9 \pm 72.0 | 257.3 \pm 56.7 | < 0.05 |
| Non adhesive platelet count | heparin | 261.1 \pm 138.3 | 198.0 \pm 125.3 | < 0.05 |
| | control | 248.6 \pm 19.6 | 172.4 \pm 27.5 | < 0.05 |
| PCV % | heparin | 36.4 \pm 3.5 | 34.4 \pm 4.0 | - |
| | control | 37.1 \pm 1.8 | 36.4 \pm 1.3 | - |

Table VIII Results of 10 units/Kg. ACTH subcutaneously in 6 rabbits given heparin intra-arterially compared to those in 7 rabbits which did not receive heparin.

| | heparin | control |
|------------|----------------|-----------------|
| percentage | 1.9 | 1.1 |
| fall | 2.4 | 5.3 |
| in | 3.6 | 6.1 |
| platelet | 13.9 | 24.0 |
| count | 19.4 | 25.1 |
| for | 25.1 | 34.6 |
| each | | 58.8 |
| rabbit | | |
| Mean | 11.1 \pm 9.9 | 22.1 \pm 20.4 |

Table IX The percentage change in platelet count following 10 units ACTH/Kg for each rabbit in the heparin and control groups.

increase platelet adhesiveness (Besterman, Myat & Travadi 1967) and be capable of inducing platelet aggregation (O'Brien 1963).

There was no correlation between the changes in plasma NEFA and the changes in platelet count or adhesiveness nor between their absolute values. The platelet aggregation which occurred appears to have been a temporary phenomenon accompanying the rise in plasma NEFA. The animals which died, did so at the time when the lipaemia resulting from ACTH would have been expected (12-24 hours) (Kellner, Hirsh & Freeman, 1960) and when fatty acids were likely to have reached basal levels again. There was no evidence of thrombosis in any of these animals and therefore it is likely that the platelet aggregation had not proceeded to thrombosis. The fact that the aggregation was reversible is suggested also by the finding that in 3 of the rabbits (the others were not tested), the platelet count had returned to the pretreatment level by the next day.

The changes in the red cells similar to that seen in "acanthocytosis" in man are of interest. In that condition, red cells have been shown to be mechanically more fragile than normal (Singer, Fisher & Perlstein 1952). The fall in the packed cell volume is probably a manifestation of this. This raises the possibility that the platelet aggregation may have been secondary to red cell damage leading to release of ADP. This change in the red cells was also temporary, and had resolved on the following day in the 3 rabbits. In "acanthocytosis", it has been shown that the defect is within the red cell and is not a function of the surrounding plasma (Salt, Wolff, Lloyd, Fosbrooke, Cameron & Hubble 1960). Clearly therefore the changes noted following ACTH are not the same, although morphologically similar. Nevertheless, the fact that there are lipid abnormalities in "acanthocytosis" - abeta lipoproteinaemia, low serum cholesterol, increased lysolethacin in the red cell and central nervous system disease (Salt et al. 1960) raises the possibility that the acute mobilisation of the fat following ACTH may have produced a similar effect on the red cell.

Heparin was given in a dose of approximately 150 units/Kg. which is a dose normally given therapeutically in man. At this dose level although there was some reduction in the amount of platelet aggregation it still occurred.

Heparin given in much higher doses (over 300 units/Kg.) inhibits platelet aggregation (Best, Cowan, & McLean, 1938, Solandt & Best 1940, Mustard, Murphy, Rowseil & Downie 1962). In the dose range applied here platelet aggregation is not prevented but the conditions producing it in all the experiments quoted have been extreme. The practical application of the present result is therefore not clear cut but it suggested that if platelet aggregation is to be prevented the dose of heparin used should be far higher than that usually used, probably of the order of 20,000 units in a 70Kg.man.

Conclusion

Platelet aggregation occurs following ACTH in rabbits, associated with very high plasma NEFA levels. No direct correlation has been demonstrated between the plasma NEFA changes and platelet adhesiveness but further investigation is indicated in view of the evidence associating NEFA and platelets. The platelet aggregation may have been due to other factors such as adrenaline, or to ACTH itself, and may have been produced via the red blood cells.

Chapter 8

Platelet Adhesiveness in the Immediate Post-Operative Period

In 1942 Payling Wright showed that there was an increase in platelet adhesiveness post operatively which was maximal along with the platelet count at about the 10th day. To explain this it was assumed that younger platelets were stickier than older platelets. The post-operative period in man is a stressful situation and Naftalin (1962) had demonstrated that there is elevation of plasma NEFA in the immediate post-operative period. If there is any relationship between plasma NEFA and platelet adhesiveness then one would expect elevation of platelet adhesiveness in the first post operative day.

Ten subjects were investigated on the first day after operation. There were 5 males and 5 females. The average age of the group was 50 years. The operations were as follows:-

| | |
|---------------------------------|---|
| gastro-enterostomy..... | 4 |
| cholecystectomy..... | 3 |
| partial thyroidectomy..... | 1 |
| resection of terminal ileum.... | 1 |
| simple mastectomy..... | 1 |

Blood was withdrawn pre-operatively from each at 9 a.m. The operation in all cases took place during the morning but the exact timing was variable. Following the operation at 2.p.m., 5 p.m. and 9 a.m. the next day further blood samples were taken. Only subjects who did not have post operative intravenous medication were used. Platelet count, platelet adhesiveness, plasma NEFA and blood sugar were estimated on each sample.

The controls consisted of a group of 7 males undergoing cystoscopy and who virtually therefore just had an anaesthetic and no significant tissue damage. These subjects were fasted until after the 5.p.m. blood sample had been withdraw. The experiment was terminated in the group at this stage. The average age of this group was 55 years.

The results on the operated group are shown in Table X and for the anaesthetic group in Table XI.

| | 9 a.m. | 2 p.m. | 5 p.m. | 9 a.m. |
|--|--------------|-----------------|-----------------|--------------|
| Initial platelet count x 10 ³ /cmm | 188.9 ± 51.4 | 199.2 ± 37.7 | 200.3 ± 49.0 | 187.1 ± 48.1 |
| Platelet stickiness % | 38.0 ± 9.5 | 48.1 ± 12.1 * | 45.3 ± 19.7 | 52.3 ± 19.0 |
| plasma NEFA MEq/l. | 634 ± 329 | 1050 ± 532 *** | 887 ± 300 *** | 682 ± 250 |
| blood sugar mg/100 m/. | 80.8 ± 7.5 | 113.1 ± 25.2 ** | 117.6 ± 30.3 ** | 107.9 ± 13.9 |

Differences from

pre-operative values:-

p < 0.05 = *

p < 0.02 = **

p < 0.01 = ***

Table X Mean results (± S.D.) for the 10 subjects undergoing major surgery between 9 a.m. and 2 p.m.

| | 9 a.m. | 2 p.m. | 5 p.m. |
|--|--------------|--------------|--------------|
| Initial platelet count x 10 ³ /cmm | 149.9 ± 27.3 | 149.9 ± 25.6 | 147.4 ± 25.1 |
| Platelet adhesiveness % | 42.4 ± 9.3 | 43.9 ± 8.0 | 45.3 ± 8.7 |
| plasma NEFA MEq/l. | 585 ± 93 | 588 ± 82 | 645 ± 196 |
| blood sugar mg/ 100 ml. | 74 ± 3.9 | 70 ± 6.1 | 75 ± 15.3 |

Table XI Mean results (± S.D.) for 7 subjects who had a cystoscopy between 9 a.m. and 2 p.m.

In the operated group the platelet count tended to rise slightly but this was not significant. The control group did not show any alteration in the platelet count. Platelet adhesiveness rose following surgery in all cases. At 2 p.m. 8 out of the 10 subjects showed an increase and this was statistically significant. At 5 p.m. platelet adhesiveness was increased in 7 out of the 10 instances while at 9 a.m. the next day, 7 out of 9 showed an increase. The mean adhesiveness at 5 p.m. and 9 a.m. was not significantly greater than the pre-operative value however. There was very much less variation in platelet adhesiveness in each individual who had only cystoscopy. The averages for this group were fairly close to one another although it is interesting that there was a tendency for platelet adhesiveness to rise as the day went on. The blood samples at 9 a.m. the next day were not taken in the control group as this would have necessitated continuing their fast until that time.

Plasma NEFA showed more uniform trends. The operated group showed a highly significant rise at 2 p.m. and 5 p.m., the highest levels being at 2 p.m. By 9 a.m. the next day the levels were not significantly above the original pre-operative level. In contrast, the subjects who had only a cystoscopy did not show any significant changes in the plasma NEFA levels.

Blood sugar was significantly elevated in the operated group at 2 p.m. and 5 p.m. This significant elevation was still present at 9 a.m. the next day. The control group did not show any significant alteration in blood sugar at any of the times tested.

Discussion

The maximal increase in plasma NEFA at 2 p.m. coincided with a significant elevation in platelet adhesiveness. There was no significant correlation between the changes however. The non significant increases in platelet adhesiveness found at 5 p.m. and 9 a.m. the next day also were not associated with corresponding plasma NEFA changes. It would appear from the results that the stimulus causing increased platelet adhesiveness was likely to have been maximal in the immediate post operative period. Bennett (1967 a) also found increased platelet adhesiveness immediately after operation. He

attributed this to stress on the basis of the accompanying changes in the white cell count. This increase in platelet adhesiveness may have more relevance to the problem of deep vein thrombosis and pulmonary embolism than the later one demonstrated by Payling Wright. It is becoming increasingly clear that, although the maximum incidence of thrombo embolic episodes is around the 10th post operative day, the primary thrombosis occurs very much earlier.

Using accurate and sensitive methods, deep venous thrombosis can be detected in the early post operative period (1-3 days). Atkins & Hawkins (1965) using I125 labelled fibrinogen, have been able to detect cases at this time. While the later increase in platelet adhesiveness demonstrated by Payling Wright may simply be in fact a function of new young platelets coming into the circulation, the increase in the first post operative day may be more significant and be the initiating thrombotic factor.

Increase in plasma NEFA post-operatively has been demonstrated previously by Naftalin (1962). The changes were not uniform in his cases but in the present series the pattern for each individual was similar. Peak levels were reached at 2 p.m. in 5 and at 5 p.m. in 5 cases. The 9 a.m. values the next day were only slightly above the baseline. Thus the stimulus to fatty acid mobilisation appears to have been maximal in the immediate post operative period (2 p.m.), the effect having declined by the next morning. The effect on glucose on the other hand was still continuing on the next day (9 a.m.). This is in accordance with the work of Ross, Johnston Welborn & Wright (1966) who showed high fasting blood sugars associated with insulin antagonism up to 72 hours post operatively. They speculated that fatty acids might have been responsible for the insulin antagonism but this obviously cannot be so since plasma NEFA levels have returned to normal within 24 hours. These workers demonstrated increased levels of hydrocortisone and growth hormone - both insulin antagonists. From their data neither of these substances looked likely contenders in causing the insulin antagonism however. Increase in catecholamine secretion could explain the fatty acid changes particularly as they were maximal immediately after the operation. This could also explain the initial elevation of the blood sugar but not its persistence when the plasma NEFA levels had returned to normal on the

following day. The possibility that the elevation of the blood sugar at 9 a.m. on the day following surgery could be due to carbohydrate ingestion must be considered. The uniformity of the changes throughout the series of patients makes this unlikely as does the fact that Ross et al. also demonstrated it. Thiopentone anaesthesia can cause insulin antagonism (Henneman & Bunker 1961) but this is not the explanation in the present investigation as there was no elevation of the blood sugar levels in the control group of patients.

Conclusions

Platelet adhesiveness was elevated in the first post operative day associated with elevation of the plasma NEFA levels. There was no direct correlation between the two. The elevation of plasma NEFA and of blood sugar immediately after surgery is thought most likely to have been a stress effect and the result of adrenal stimulation. The persistence of the elevation of the blood sugar was thought to represent insulin resistance but the reason for this could not be explained.

Chapter 9Effects of cigarette smoking

The expectation of finding elevation of platelet adhesiveness in the first post operative day, based on the knowledge that plasma NEFA levels were elevated at this time, was fulfilled. Cigarette smoking is also known to elevate plasma NEFA levels (Kershbaum, Bellet, Dickstein & Feinberg 1961) and is thought to elevate platelet adhesiveness (Ashby, Dalby & Millar, 1965). In view of the reported importance of cigarette smoking predisposing to ischaemic heart disease (Brit.Med.J. 1966) the effects of cigarette smoking both on platelet adhesiveness and serum lipids were considered to be of great interest.

Method

Twelve patients (8 males and 4 females) from a general medical ward who habitually smoked 10 or more cigarettes daily were investigated. They were aged 37-67 years with a mean age of 55 years. Their diagnoses were:-

| | |
|--------------------------------|---|
| ischaemic heart disease..... | 3 |
| peripheral vascular disease... | 3 |
| hypertension..... | 1 |
| idiopathic diarrhoea..... | 1 |
| simple goitre..... | 1 |
| rheumatic heart disease..... | 1 |
| epilepsy..... | 1 |
| pneumonia..... | 1 |

The patients fasted overnight and remained at rest in bed throughout the experiment. Two basal venous blood samples were taken 20 minutes apart. The patients were then instructed either to smoke two of their usual brand of cigarettes during a 15 minute period or to sham smoking by puffing the unlit cigarette for the same length of time. A third blood sample was taken at the end of the 15 minute period, and three further samples at 10 minute intervals. Within the next few days the experiment was repeated

with sham smoking substituted for the smoking or vice versa. 7 patients smoked in the first experiment and 5 in the second. For determination of the individual non-esterified fatty acids the NEFA fraction was separated by thin layer chromatography using plates coated with "adsorbosil" impregnated with rhodamine B. The solvent system used with hexane/diethyl ether/methanol/acetic acid in a proportion of 90;20;3;2 V/V. The NEFA band was scraped from the plate and the fatty acids were eluted with ether and methylated with methanol and sulphuric acid. The fatty acids were separated by gas liquid chromatography on a panchromatograph column containing 15% polyethylene glycol adipate on Gas Chrom.P. Serum cholesterol was measured by a modification of the Technicon Autoanalyser Method N24P which basically involves a colour reaction between cholesterol, ferric chloride and sulphuric acid originally described by Zak, Dickenman, White, Burnett & Cherney (1954). Serum triglyceride was measured by the method of van Handel & Zilversmit (1957) which uses triolein standards.

Results

The results of the 12 smoking tests are shown in Table XII and those of the 12 sham smoking tests in Table XIII.

There was a significant rise in the mean plasma NEFA level after smoking, reaching a maximum 10 minutes after the end of the smoking period and falling again to the basal level over the succeeding 20 minutes. While the plasma NEFA level increased to a variable extent in every case after smoking, after sham smoking the plasma NEFA level rose in 5 cases, was unchanged in 3 and fell in 4. The small increase after sham smoking was not statistically significant.

Both smoking and sham smoking were followed by a significant increase in the concentration of oleic acid in the NEFA fraction, and a significant fall in the concentration of palmitic acid. This effect was more notable after sham smoking than after smoking and did not correlate well with the changes in total plasma NEFA. The other fatty acids in the NEFA fraction showed a similar tendency for an increase in unsaturated at the expense of saturated fatty acids, but the individual changes were not significant.

There was no significant change in serum cholesterol or triglyceride levels

in either group.

Smoking was followed by a small but significant increase in blood glucose. This happened in every case and showed no tendency to return to the fasting level. There was no change after sham smoking.

There was a significant transient rise in the total platelet count after smoking, but no change after sham smoking. On the other hand, there was a mean rise in platelet adhesiveness in both smoking and sham smoking groups. There was an increase in 9 of the 12 sham smoking tests, and in this group the mean rise was significant. The changes after smoking were more variable, a rise in 7 cases and a fall in 5 cases, and the mean rise was not statistically significant.

There was no significant difference between the 6 patients with arterial disease and the others, in basal serum lipid levels, blood glucose and platelet adhesiveness or in the magnitude of the responses to smoking.

There was no correlation between basal platelet adhesiveness and basal levels of either glucose or NEFA.

In the sham smoking group it was evident that there was a good correlation between the percentage rise in NEFA and the rise in platelet adhesiveness as shown in Fig.4. $\% \text{ rise} = \frac{45 \text{ minute level} - \text{Mean basal level}}{\text{Mean basal level}} \times 100$

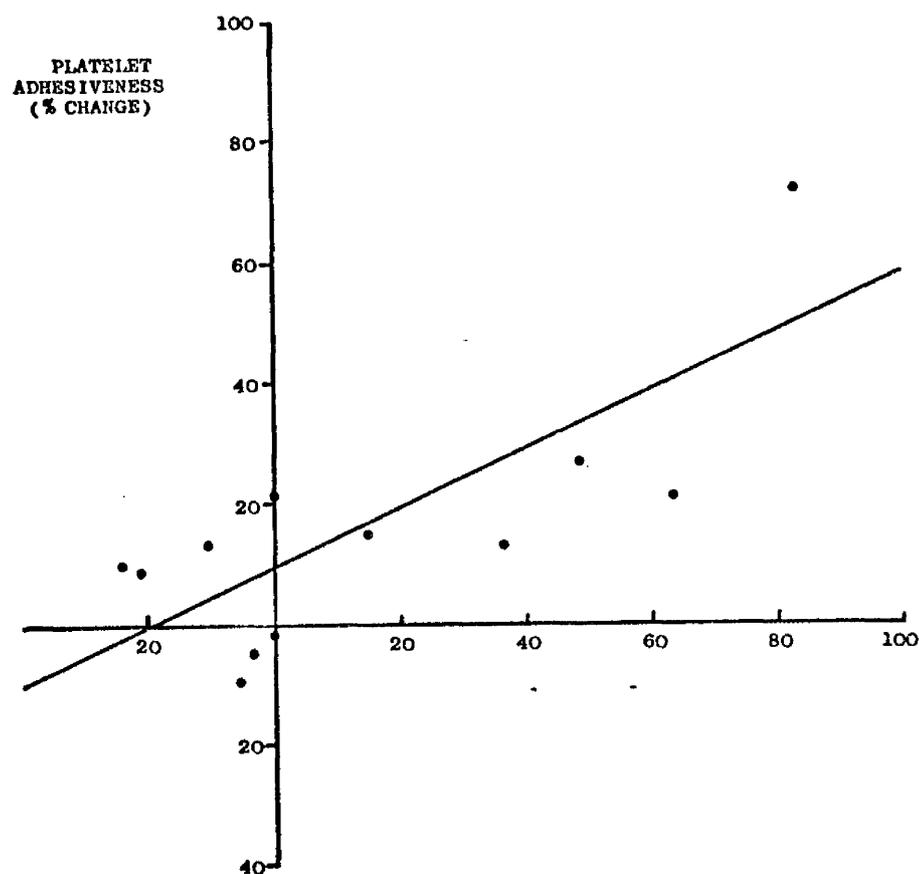


Figure 4.

Correlation between percentage changes in plasma NEFA and platelet adhesiveness after sham smoking.

$$y = 0.51x + 9.19 \quad (r = 0.7091)$$

This correlation was significant at the 1% level ($r=0.709$). There was a similar but less significant correlation between percentage changes in NEFA and platelet adhesiveness after smoking ($r=0.584$, $p < 0.05$) as shown in Fig. 5.

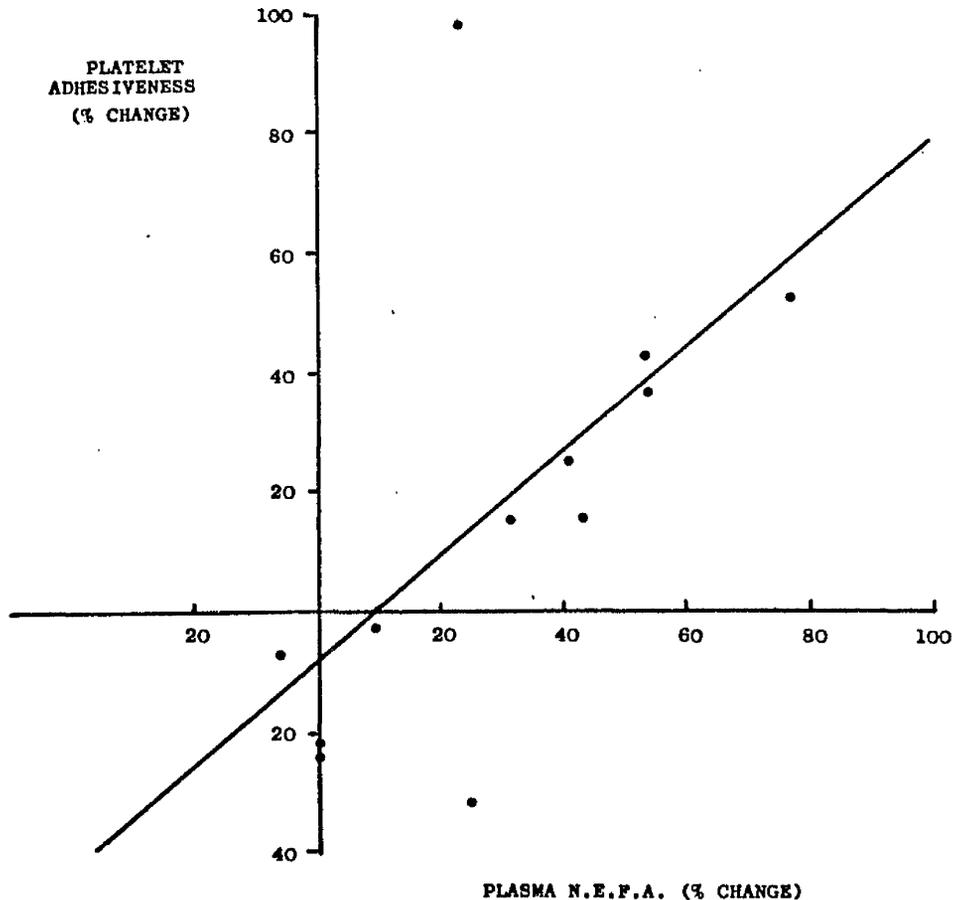


Figure 5. Correlation between percentage changes in plasma NEFA and platelet adhesiveness after smoking.

$$y = 0.86x - 8.22 \quad (r=0.5838)$$

In the smoking group however, there was a rise in glucose and Fig 6. illustrates the negative correlation between changes in blood glucose and platelet adhesiveness ($r=0.701$, $p < 0.02$).

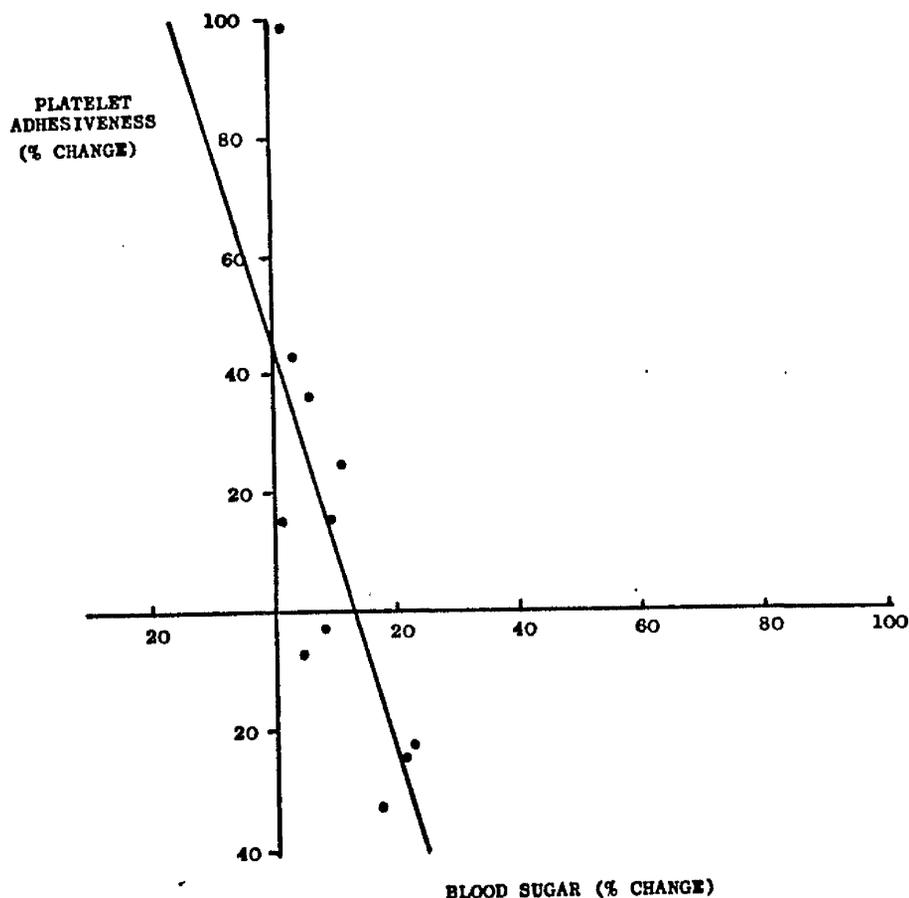


Figure 6. Correlation between percentage changes in blood glucose and platelet adhesiveness after smoking.

$$y = -3.6X + 46 \quad (r = -0.7011)$$

The relationship between the % change in platelet adhesiveness (y) and % changes in NEFA (x_1) and blood glucose (x_2) after smoking can be expressed as follows:-

$$y = 0.43x_1 - 3.0x_2 + 30.5$$

Using this formula, the correlation between the actual and predicted values for platelet adhesiveness is significant at the 1% level ($r=0.726$).

A correlation between changes in plasma NEFA and changes in platelet adhesiveness, demonstrated in the sham smoking group has never been shown before. The results of this investigation were therefore published (Murchison & Fyfe, 1966). The finding of significant elevation of

| | Basal | | | After smoking | | | |
|--------------------------------------|---------------------------|---------------------------|-------------------------|-----------------------------|------------------------|---------------------------|--|
| | 0 min. | 20 min. | 35 min. | 45 min. | 55 min. | 65 min. | |
| Plasma - NEFA (μ Eq. per l.) | 395 [±] 149 | 393 [±] 149 | 453 [±] 150*** | 496 [±] 164** | 442 [±] 96 | 399 [±] 95 | |
| Oleic acid (%) | 39.9 [±] 3.8 | 39.7 [±] 3.6 | 40.8 [±] 3.5 | 41.7 [±] 3.0* | 40.2 [±] 4.9 | 40.8 [±] 4.8 | |
| Palmitic acid (%) | 29.5 [±] 1.9 | 29.8 [±] 2.4 | 28.5 [±] 1.9 | 27.7 [±] 1.7* | 28.4 [±] 3.4 | 28.4 [±] 3.0 | |
| Blood-glucose (mg. per 100 ml.) | 68.2 [±] 8.6 | 72.6 [±] 9.2 | 73.6 [±] 9.2 | 76.7 [±] 9.2* | 76.7 [±] 8.0* | 76.9 [±] 7.1 * | |
| Platelet count (per c.mm) | 157600 [±] 32600 | 155500 [±] 41800 | . . . | 166300 [±] 35000** | . . . | 157100 [±] 34700 | |
| Platelet adhesiveness (%) | 35.5 [±] 14.1 | 36.9 [±] 12.2 | . . . | 39.6 [±] 12.2 | . . . | 35.1 [±] 13.0 | |

Table XII Mean ([±] S.D.) of changes after cigarette smoking.

differences significant at P: <0.05 *
 <0.01 **
 <0.001 ***

| | Basal | | After sham smoking | | | |
|---------------------------------|---------------------------|---------------------------|------------------------|---------------------------|------------------------|---------------------------|
| | 0 min. | 20 min. | 35 min. | 45 min. | 55 min. | 65 min. |
| Plasma NEFA (MEq. per l.) | 369 [±] 76 | 359 [±] 67 | 381 [±] 71 | 403 [±] 82 | 386 [±] 83 | 369 [±] 89 |
| Oleic acid (%) | 37.5 [±] 3.5 | 39.8 [±] 4.6 | 40.5 [±] 5.2* | 41.4 [±] 4.8* | 40.5 [±] 4.7* | 41.0 [±] 5.7** |
| Palmitic acid (%) | 30.7 [±] 3.5 | 29.8 [±] 2.5 | 29.7 [±] 3.2 | 27.8 [±] 2.3* | 28.8 [±] 1.7* | 29.4 [±] 2.2 |
| Blood-glucose (mg. per 100 ml.) | 68.6 [±] 8.5 | 71.3 [±] 8.6 | 70.8 [±] 9.0 | 70.4 [±] 8.4 | 70.1 [±] 7.2 | 71.4 [±] 7.4 |
| Platelet count (per c.mm) | 165000 [±] 43800 | 153000 [±] 38400 | . . | 161400 [±] 47500 | . . | 160400 [±] 50100 |
| Platelet adhesiveness (%) | 41.0 [±] 8.3 | 43.7 [±] 7.9 | . . | 48.3 [±] 7.3 * | . . | 47.3 [±] 11.1 |

Table XIII Mean ([±] S.D.) of changes after sham smoking

Differences significant at P: <0.05 *
 <0.01 **
 <0.001 ***

platelet adhesiveness following sham smoking was a surprise. Although the rise in plasma NEFA was not statistically significant it did rise in 5 out of 12 instances in the sham smoking group and there was a significant change in the proportions of oleic and palmitic acids. The fact that the changes followed a steady basal period suggests that the effects were due to sham smoking rather than repeated venepuncture and might possibly have been due to the embarrassment of puffing an unlit cigarette. Sham smoking has been previously shown to produce physiological changes by Irvine and Yamamoto (1963) in their investigation of the effects of cigarette smoking on cardiac output.

In the smoking experiment, the correlation between the changes in plasma NEFA and platelet adhesiveness although still apparent appeared to be partially obscured by the rise in blood sugar which correlated negatively with the platelet adhesiveness changes. The previous experiments involving ACTH in rabbits and surgery in man showed increases in blood sugar accompanying the plasma NEFA and platelet adhesiveness changes. The lack of correlation in these experiments between changes in plasma NEFA and platelet adhesiveness may not have been found because of this. Applying the equation obtained in this experiment to those other experiments, however, showed that the equation did not hold. The finding of a correlation does not necessarily imply a cause and effect relationship. It may be for example that catacholamine release could have caused effects, the plasma NEFA, blood sugar and platelet adhesiveness changes merely being 'indicators' of its release. The increase in proportion of unsaturated fatty acids at the expense of saturated fatty acids is another complicating factor if one accepts the evidence that unsaturated fatty acids may protect platelets from the aggregating effects of saturated acids (Kerr, Pirrie, MacAulay & Bronte-Stewart, 1965). This type of change in the fatty acid pattern is likely to have followed the mobilisation of fat after ACTH in the rabbits and after surgery in man, because mobilisation of fatty acids tends to make the plasma NEFA pattern more like that of depot fat. Mobilisation of fat by various stimuli has this effect (Rothlin, Rothlin & Wendt, 1962; Wood Schlierf & Kinzell 1965).

It is quite clear that in all the experiments conducted so far, an increase in platelet adhesiveness has accompanied an increase in plasma NEFA levels. There has been no direct correlation between the two apart from in the smoking/sham smoking experiments. This may be because there is no relationship between the changes, or if there is, because the correlation has been obscured by the alteration in the fatty acid pattern or by changes in blood sugar. The former is an unlikely explanation since a correlation has been demonstrated in the sham smoking experiment in which the fatty acid pattern changed significantly.

The negative correlation between the rise in blood sugar and the change in platelet adhesiveness is contrary to the work of Bridges, Dalby, Millar & Weaver (1965) who found an increase in adhesiveness associated with the rise in blood sugar following oral glucose. They did not show any direct correlation between the two however. In this situation one would expect the plasma NEFA levels to have fallen and the pattern to have become more saturated. Could it have been that this relative increase in plasma NEFA saturation was responsible for the rise in platelet adhesiveness? The present situation in which endogenous glucose is being mobilised is quite different from the changes following oral glucose. The rise in blood sugar following smoking has been described before by Lundberg & Thyselius-Lundberg (1931) and by Rehder & Roth (1959) who suggested that it may have been a stress effect. In the present series it can be attributed to smoking per se as it did not occur in the control group.

The finding of Ashby, Dalby & Millar (1965) of a significant increase in platelet adhesiveness following smoking has not been confirmed. Although 7 cases showed a considerable rise in platelet adhesiveness following smoking, in 5 others in whom there was a marked rise in blood sugar, the platelet adhesiveness fell. This effect was most evident in two patients who felt nauseated at the end of the smoking period and who showed the largest increases in blood sugar in association with delay in the plasma NEFA rise. Since Ashby et al. (1965) only measured platelet adhesiveness it is not possible to explain the discrepancy between their results and the present ones. It may be however that in the non fasting state the blood

sugar response to smoking is less striking.

An increase in the platelet count after smoking has been reported by Grassi & Caltabiano (1956). The mechanism is obscure but may be the result of adrenaline release (Vaughan-Jones, Ingram, McClure 1963). The present results confirm the increase in platelet count following smoking.

Conclusion

Sham smoking was followed by a significant rise in platelet adhesiveness and in the proportion of unsaturated fatty acids in the plasma NEFA. The changes in the plasma NEFA levels correlated with the changes in platelet adhesiveness. This correlation was also evident after smoking but in this group there was also a significant increase in blood sugar and this correlated negatively with the platelet adhesiveness changes. There was also a significant rise in the platelet count following smoking.

Chapter 10

Effects of Exercise

Severe exercise has been reported to cause an increase in platelet adhesiveness (Ikkala, Myllyla & Sarajas 1966 , Finkel & Cumming 1965) while less severe but more prolonged, exercise such as a walk from London to Brighton produces a reduction in platelet adhesiveness (Pegrum, Harrison, Shaw, Haselton & Wolff, 1967). When standardising a method of measuring platelet adhesiveness it is obviously important to determine the effect of exercise and how long this effect lasts. In the previously described experiments into the effects of surgery and cigarette smoking all the subjects were at rest for several hours prior to commencement of the experiments. Clearly, particularly when outpatients are involved, the necessity of resting and the time required to reach basal conditions must be determined in order to get comparable results with the minimum amount of inconvenience.

Severe exercise is known to increase the turnover of plasma NEFA, producing an initial depression of the plasma level followed by a post exercise rebound (Friedberg, Sher, Bogdonoff & Estes 1963; Wood Schlierf & Kinsell 1965). Also, unaccustomed exercise is thought to be a factor leading to myocardial infarction (Plotz, 1957). It is possible that fatty acid metabolism and platelet deposition could be involved in the mechanism of this association. The changes in platelet adhesiveness during and after exercise in association with the fatty acid changes were therefore investigated.

Method

Nine young healthy male subjects took part in the investigation. One subject was unable to complete the exercise schedule because of exhaustion and vomiting. This patient was therefore excluded because the results on him were incomplete. The average age of the remaining subjects was 25 years (range 19 - 30 years).

All the subjects were fasting. On arrival at the hospital they were

placed horizontal on a couch and allowed to rest for at least 20 minutes. Two basal blood samples (1 & 2) were then obtained 20 minutes apart, prior to starting the periods of exercise. The subjects exercised, without moving from the horizontal position, using an electrically balanced bicycle ergometer. They exercised against a resistance of 1500 kilopond-metres for 5 minutes after which a further blood sample (no.3) was taken. A further period of exercise followed, lasting 5 minutes again, but on this occasion against a resistance of 750 kilopond-metres. Blood was withdrawn towards the end of this period of exercise while the subjects were still exercising. (sample 4). Thereafter a further 4 samples (no.5-8) were taken at 5 minute intervals. Platelet adhesiveness was measured on each sample as previously described except that the measurement was commenced 3 minutes after withdrawal of the blood. P.C.V., plasma NEFA and blood sugar were also measured in all blood samples.

Results

The 8 subjects who managed to complete the exercise programme were all exhausted, breathless and sweating profusely at the end of it. All however recovered quickly (in about 5 minutes) from the effects.

The average results for the 8 subjects are shown in Table XIV.

Platelet adhesiveness rose in 6 out of 8 cases immediately after the first period of exercise. The average rise was however not statistically significant. After the second period of exercise, platelet adhesiveness was elevated above the mean basal level in all cases and the average increase was statistically significant. ($p < 0.01$). In the subsequent samples the average platelet adhesiveness returned gradually to the basal levels. One value was eliminated by Chauvenet's criterion from the results in sample 6 as it was unrealistic and would have unduly distorted the average result. This value was one of 60.3% which was an isolated high value among the other 7 values for that subject which ranged from 18.2 to 30.7%.

The platelet count rose in every case following exercise. This increase was significant at 1% level after the first period of exercise, at the 0.1% level after the second period and at the 5% level 5 minutes after termination

| Blood sample Number | 1 | Exercise 1500 K.p.m for 5 min | | | Exercise 750 K.p.m/4 for 5 min | | | 5 min. | 10 min. | 15 min. | 20 min. |
|---|---------------|-------------------------------|--------------|--------------|--------------------------------|--------------|--------------|--------------|---------|---------|---------|
| | | 2 | 3 | 4 | 5 | 6 | 7 | | | | |
| time after exercise (min) | | | | | | | | | | | |
| platelet adhesiveness % | 22.6 ± 5.5 | 21.3 ± 4.9 | 26.1 ± 5.3 | 32.7 ± 6.3 | 25.6 ± 5.2 | 26.8 ± 5.7 | 25.4 ± 5.4 | 23.6 ± 4.5 | | | |
| platelet count $\times 10^3/\text{cmm}$ | 210.6 ± 40.2 | 202 ± 35.7 | 241.9 ± 44.6 | 258.5 ± 56.7 | 241.0 ± 55.3 | 221.4 ± 52.3 | 206.1 ± 53.1 | 198.8 ± 49.1 | | | |
| P.C.V.% | 44.20 ± 1.96 | 44.10 ± 2.00 | 48.50 ± 1.94 | 47.80 ± 2.46 | 45.60 ± 2.30 | 44.00 ± 2.32 | 44.60 ± 2.51 | 43.80 ± 1.64 | | | |
| Plasma NEFA MEq/l. | 398.3 ± 165.1 | 395.1 ± 170.6 | 261.9 ± 87.1 | 306.5 ± 44.4 | 351.1 ± 54.2 | 320.9 ± 86.3 | 319.9 ± 42.3 | 353.4 ± 86.9 | | | |
| blood sugar mg% | 68.9 ± 8.8 | 71.1 ± 7.4 | 69.9 ± 8.0 | 70.1 ± 10.0 | 71.9 ± 9.6 | 69.5 ± 10.9 | 68.8 ± 9.4 | 60.0 ± 8.7 | | | |

* $p < 0.05$
 ** $p < 0.01$
 *** $p < 0.001$
 ⊕ corrected (see text)

TABLE XIV Mean results (± S.D.) for 8 subjects who exercised for 5 minutes between samples 2 and 3 and between samples 3 and 4.

of exercise. The platelet count had returned to the basal level 15 minutes after the exercise.

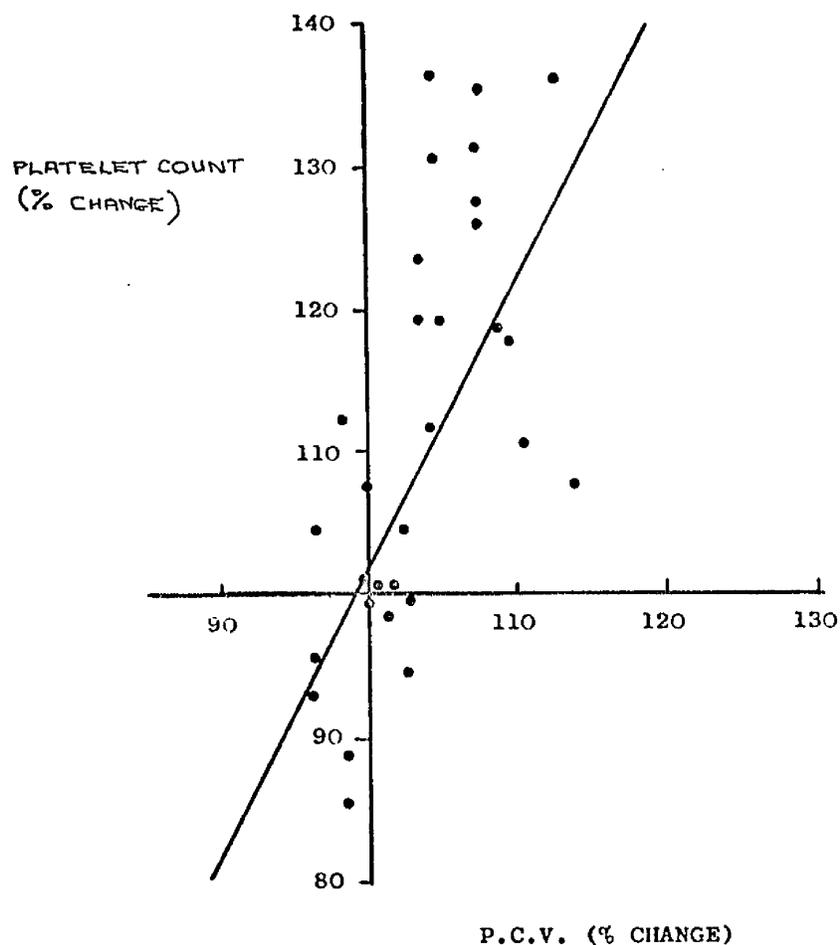


Figure 7. Correlation between the percentage changes in platelet count and packed cell volume (P.C.V.) following exercise

$$y = 2.0x - 98.4 \quad (t = 0.657; p < 0.001)$$

The packed cell volume (P.C.V.) increased in all cases following both periods of exercise, returning to the basal level within 10 minutes of stopping the exercise.

The changes in the P.C.V. were statistically significant and correlated significantly with the changes in platelet count ($r = 0.657$, $p < 0.001$)

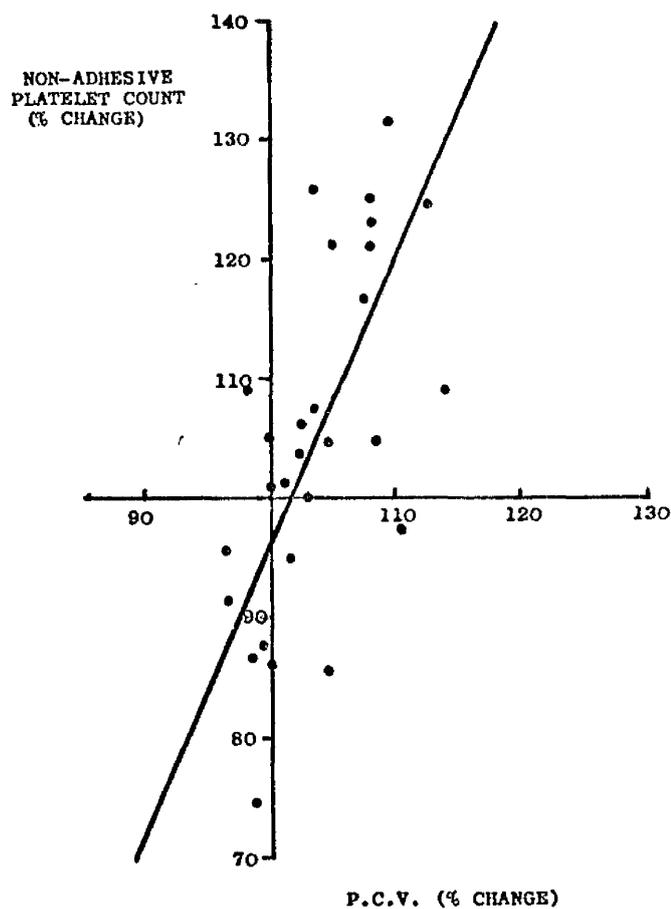


Figure 8. Correlation between the percentage changes in "non-adhesive platelet count" and packed cell volume (P.C.V.) following exercise.

$$y = 2.5x - 154.4 \quad (r = 0.610; p < 0.001)$$

and with the changes in the non adhesive platelet count ($r = 0.610$, $p < 0.001$) throughout the experiment.

There was no correlation with the changes in adhesive platelet count

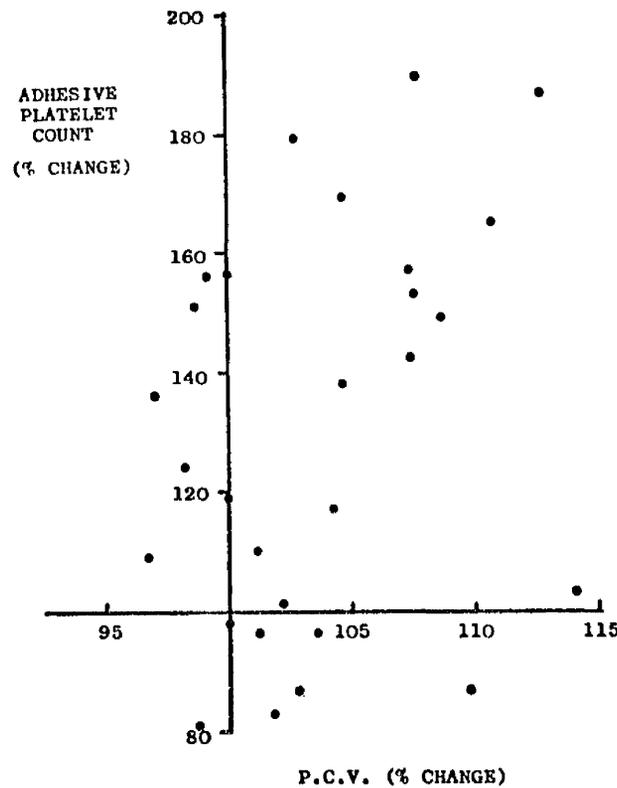


Figure 9. Relationship between the percentage changes in "adhesive platelet count" and packed cell volume (P.C.V.) following exercise.

($r = 0.066$) or platelet adhesiveness ($r = 0.243$). These correlations are shown in figures 7 to 10.

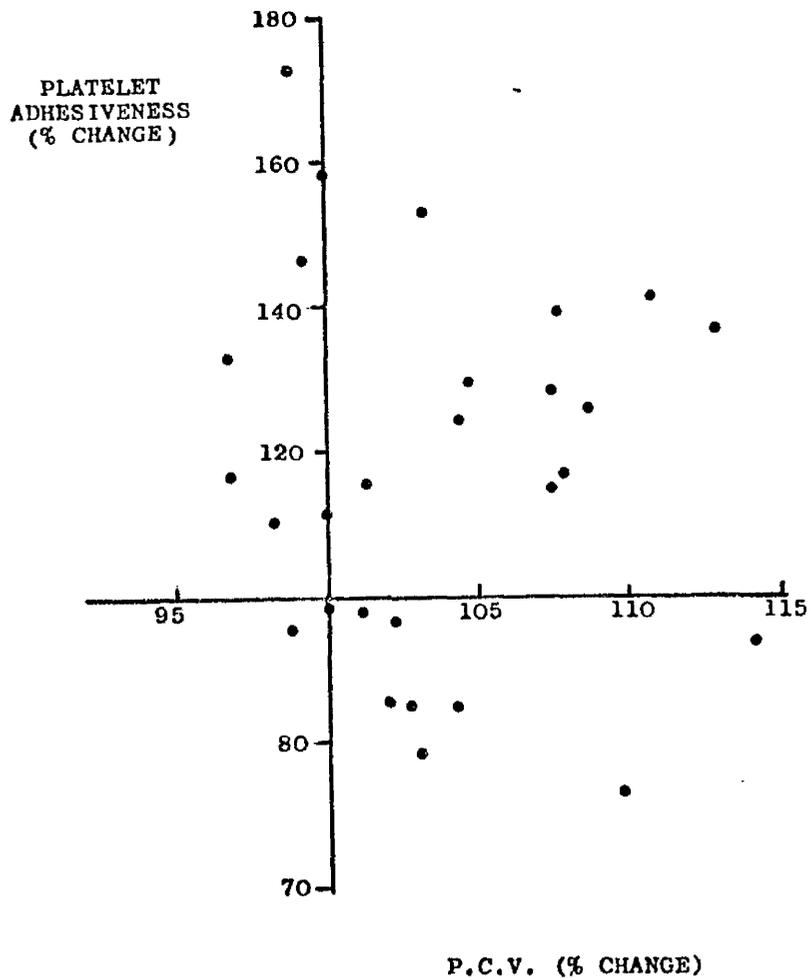


Figure 10. Relationship between the percentage changes in platelet adhesiveness and packed cell volume (P.C.V.) following exercise.

Plasma NEFA levels were suppressed significantly following the first period of exercise ($p < 0.01$). The average level was still low after the second period but the difference from the basal level was not significant. There was no evidence of any 'rebound' elevation of the plasma NEFA level following exercise. Indeed the levels tended to remain slightly below the mean basal level. There was no correlation between the changes in platelet adhesiveness and the changes in plasma NEFA.

The blood sugar levels did not alter significantly.

Discussion

Ikkala, Myllyla & Sarajas (1966), and Finkel & Cumming (1965) in their investigations into the effect of exercise on platelet adhesiveness found that exercise produced an increase in platelet count. The former workers and their assistants had previously found this in 1961 & 1963. (Sarajas, Konttinen & Frick 1961; Ikkala, Myllyla & Sarajas 1963). Biggs, MacFarlane & Pilling (1947) also demonstrated an increase but following this both Gerheim & Miller (1949) and Sloan & Allardyce (1955) found decreases in the platelet count immediately following exercise. Both these groups found that the white cell count was elevated in association with the decreased platelet count. The reason for this finding is obviously that platelets were being lost. There is no mention in these papers of special precautions, such as siliconising of equipment, taken to preserve platelets. The most obvious possibility is that the platelets were adhering to equipment. This would be more likely to occur after exercise because of the likely increase in adhesiveness of platelets. The cause of the increased platelet counts after exercise may be that platelets are picked up from pulmonary and peripheral capillaries by the dynamic increase in the circulation. This is supported by Sarajas, Konttinen & Frick (1961) who found that the increases in cardiac output and relative circulation velocity were commensurate with those of the platelet count. Although there is a correlation between the increase in platelet count and the increase in P.C.V., the fact that the slope of the line is greater than 45° shows that the increase in platelet count is greater than one would expect simply from P.C.V. changes. The same goes for the non-adhesive platelet count. This means that the counts have risen even when the effects of haemoconcentration have been taken into account. The fact that there is no correlation between the P.C.V. and the adhesive platelet count or platelet adhesiveness is at first surprising. This is because the two lines expressing the correlation between P.C.V. and platelet count and between P.C.V. and non adhesive platelet count are not parallel, the increase in the latter being greater. The relatively greater increase in non adhesive platelet count associated with P.C.V. means that the P.C.V. changes are not responsible for the increase in platelet

adhesiveness following exercise.

It will be noted that the levels of platelet adhesiveness in this study are lower than in other experiments. This is because the interval between venepuncture and measurement of adhesiveness was 3 minutes (c.f. the study on the effect of variation of this time interval). This timing was chosen so that different manoeuvres such as venepuncturing and the pipetting of blood would be out of phase within the design of this experiment.

The increase in platelet adhesiveness following acute exercise has been described previously by Ikkala et al. (1966) and Finkel & Cumming (1965). The investigation of Pegrum et al. (1967) into the effects of walking from London to Brighton and in which there were many uncontrolled variables is not comparable. The increase in platelet adhesiveness was associated with depression of the plasma NEFA levels suggesting that the relationship between the two demonstrated in the smoking/sham smoking experiment is not valid. There are, however, two complicating features. Firstly, the exercise was very severe and stress would undoubtedly have lead to catecholamine release. This could have effected the platelets in view of the work of Besterman, Myat & Travadi (1967) and of O'Brien (1963). Secondly, since the depression of plasma NEFA is due to increased utilisation of fatty acids, the proportion of saturated fatty acids would have increased in proportion to the unsaturated fatty acids (Wood, Schlierf & Kinsell, 1965). It could be argued that this relative increase in saturated fatty acids might have caused the increased adhesiveness in view of the previously cited in vitro work incriminating saturated rather than unsaturated fatty acids in producing platelet aggregation. This however would appear unlikely in view of the fact that the correlation in the smoking/sham smoking experiment was demonstrated in spite of an increase in the proportion of unsaturated fatty acids which could be argued from the same in vitro work to have a protective effect on platelets.

The significance of the increase in platelet adhesiveness after exercise and its relationship to the belief that coronary thrombosis is more common after unaccustomed exercise is not clear. The increase in platelet adhesiveness could be the factor which tips the balance in favour of

thrombosis in a susceptible subject. Exercise is known to increase other coagulation factors such as factors XII and VIII and to shorten the whole blood clotting time and the partial thromboplastin time (Iatridis & Ferguson 1963) although this effect on the partial thromboplastin time has been disputed by Egeberg (1963). On the other hand, exercise also increases fibrinolysis (Iatridis & Ferguson 1963) and any hypercoagulability may thus be counterbalanced. The relationship of platelet adhesiveness to thrombosis and the coagulation system is unknown. One might expect that surface active factors (XI and XII) would be related to platelet adhesiveness in view of their depending on a common stimulus. This is suggested by the work of Sharp (1958), Waaler, (1959) & Jurgens (1962) but it is disputed by Barth, Kommerell & Pfleiderer (1966) who showed that surface activation did not accelerate platelet aggregation using platelet rich plasma in the Payling Wright system. The latter paper would appear to be most relevant to the present investigations since the system used was similar. However their procedure was fairly complex and one feels that there must have been quite a delay between venepuncture and the carrying out of the tests so that the possibility of spontaneous platelet aggregation (which occurs after 40 - 60 minutes) lowering the initial count and this affecting the control must be raised. A further anomaly in that investigation is that decreases in adhesiveness are described when increases occur.

Platelet adhesiveness had returned to around the basal levels by 20 minutes following cessation of exercise. In order that effects due to exercise can be eliminated when measuring platelet adhesiveness, subjects should be rested for at least 20 - 30 minutes before the blood sample is withdrawn. This is obviously of considerable practical importance if measurements are to be comparable.

Chapter 11

Effects of starvation in obese subjects

Obesity is acknowledged as being a factor predisposing to ischaemic heart disease (Dawber, Kannell, Revotskie & Kagan 1962). Platelet adhesiveness in this condition would therefore be of particular interest. In addition, rapid weight loss in diabetics is said to predispose to thrombosis (Beckett & Lewis 1960). Platelet adhesiveness was therefore investigated in obese subjects who had agreed to lose weight rapidly by fasting for 10 days, to determine whether platelets are stickier than normal in obesity, and if fasting is associated with any increase in platelet adhesiveness.

Method

Sixteen subjects, 3 males and 13 females, were investigated. Two of the females underwent a second period of starvation, raising the total number of episodes of fasting to 18.

Following admission to hospital, the patients remained on a diet similar to that which they had been having at home. In 10 instances this was unrestricted and in 8 it was a 600 calorie diet. This diet was continued for 3 to 7 days, after which the patients were starved completely for 10 days. Only non-nutritious fluids were allowed, such as water, black coffee or unsweetened lemon tea. Vitamin supplements were not given and no drugs were administered. During the refeeding period of 24-48 hours before they left hospital, they were given a 600 calorie diet. This was continued at home and the patients were seen again after 1 - 4 weeks, in most cases after 2 weeks.

Blood samples were taken after an overnight fast on at least 3 occasions in most subjects during the basal period. Further samples were taken on day 2, 4 or 5, 6 or 7, and 9 of the fast. Samples were also taken after an overnight fast following refeeding before the patients left hospital, and again at the follow-up examination.

Platelet count, platelet adhesiveness, P.C.V., plasma NEFA levels and pattern, blood sugar, serum cholesterol and serum triglycerides were

measured on all samples by the methods previously described. Platelet adhesiveness was measured 20 minutes following venepuncture. Double extraction of plasma NEFA was carried out in 8 out of the 18 instances. The patient's weight, urinary ketones and fluid balance were also measured.

Urinary ketones were assessed using Ames Acetest tablets.

Fluid intake and output were charted throughout the period in hospital from 8 a.m. each day.

Results & Discussion

Table XV shows the results of the measurement carried out on the blood samples. The results are shown as means and standard deviations. The weight and the fluid balance changes are shown in Fig.11.

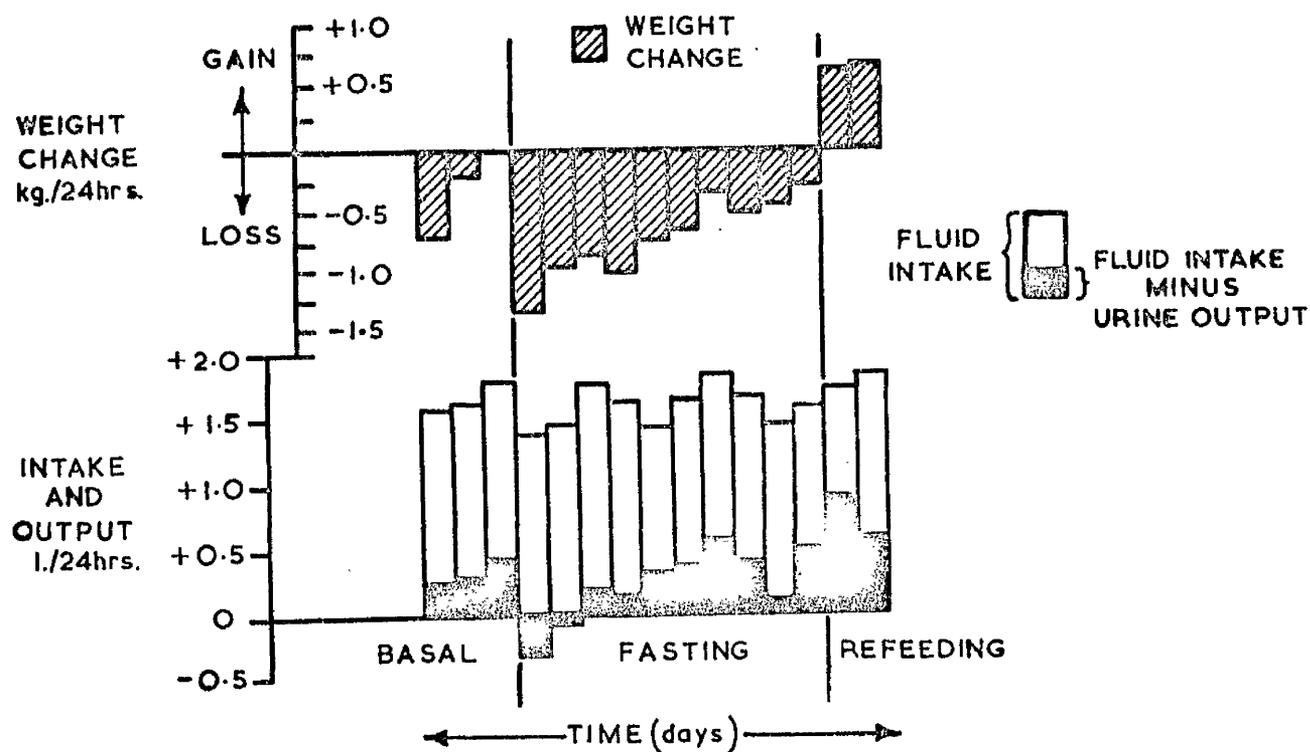


Figure 11. Mean changes in body weight and fluid balance during and after 10 days starvation.

| | | FASTING | | | | | | | | |
|-------------------------------|----------------|-----------------|-----------------|-----------------|----------------|-----------------|----------------|--|--|--|
| | mean basal | day 2 | day 4 - 5 | day 6 - 7 | day 9 | refeeding | late | | | |
| platelet adhesiveness % | 39.2 ± 9.3 | 37.0 ± 14.2 | 40.0 ± 16.6 | 40.3 ± 15.4 | 39.1 ± 13.3 | 34.4 ± 11.5 | 41.3 ± 14.6 | | | |
| platelet count /cmm | 188090 ± 43210 | 197560 ± 50210* | 200190 ± 45080* | 208390 ± 47110* | 191990 ± 39860 | 182920 ± 43760 | 211190 ± 57440 | | | |
| plasma NEFA double extraction | 503 ± 143 | 894 ± 279** | 836 ± 232** | 930 ± 226*** | 823 ± 187** | 712 ± 246 | 453 ± 176 | | | |
| plasma NEFA single extraction | 545 ± 126 | 865 ± 416** | 885 ± 349*** | 1118 ± 435*** | 965 ± 361*** | 799 ± 303 | 538 ± 181 | | | |
| fasting blood sugar mg/100ml | 73.5 ± 11.0 | 56.0 ± 8.9*** | 50.8 ± 11.3*** | 54.0 ± 7.0*** | 59.8 ± 9.8*** | 77.8 ± 12.4 | 74.9 ± 14.3 | | | |
| serum cholesterol mg/100ml | 245.8 ± 39.1 | 250.9 ± 41.7 | 272.6 ± 50.2** | 273.3 ± 63.8* | 248.0 ± 43.8 | 209.5 ± 37.0*** | 243.2 ± 45.2 | | | |
| serum triglyceride mg/100ml | 84.5 ± 19.7 | 88.9 ± 18.3 | 108.7 ± 18.3*** | 104.9 ± 21.3** | 88.0 ± 40.5 | 76.3 ± 15.9 | 88.2 ± 31.0 | | | |
| P.C.V. % | 43.6 ± 3.9 | 44.8 ± 4.0* | 46.1 ± 5.1** | 45.5 ± 4.0*** | 44.9 ± 5.3 | 43.4 ± 4.6 | 42.1 ± 4.7* | | | |

* p < 0.05
 ** p < 0.01
 *** p < 0.001

TABLE XV Mean results (± S.D.) for 18 episodes of starvation in 16 subjects, except in the cases of plasma NEFA (double extraction) which covers only 8 episodes of fasting and serum triglyceride from which the results of 2 hyperglycaemic subjects have been excluded.

There was a diuresis which occurred immediately on fasting and which gradually subsided during the first week. The weight changes during this period paralleled the fluid balance changes and on refeeding there was an increase in weight corresponding to the fluid retention. However, by the time of the follow-up examination 2 weeks after the fast when the fluid balance changes would have corrected themselves a significant amount of weight had been lost (7.1 ± 2.4 Kg).

The pattern of change for serum cholesterol, serum triglyceride, P.C.V. and platelet count on fasting were also similar to the fluid balance changes and it would appear likely that they merely reflect plasma volume changes. The changes in these measurements on refeeding however cannot all be explained on this basis.

The serum cholesterol fell out of proportion to the changes in fluid balance and P.C.V. on refeeding but the reason for this is not clear. It should be noted that the basal serum cholesterol levels were within normal limits in all but 3 cases.

While the serum triglyceride levels paralleled the P.C.V. and fluid balance changes in these subjects with levels within normal limits, there were 2 subjects with high triglyceride levels (307 and 238 mg/100 ml.). In these subjects the serum triglyceride fell drastically on starvation probably because they were likely to have had the commonest form of hypertriglyceridaemia namely carbohydrate induced (Ahrens, Hirsch, Oette, Farquhar & Stein, 1961). These subjects did not show any evidence of this unusual pattern of change affecting the pattern of change in platelet adhesiveness.

The P.C.V. after its initial rise, fell during the latter half of starvation through the refeeding period and again at the follow-up examination. Erythropoietin lack which has been shown in rats to occur on protein deprivation (Ito & Reissman 1966) may be a factor producing this fall during the fast, while on refeeding fluid retention is a further factor. The significant fall noted at the follow up examination may be due to blood loss resulting from repeated venepuncture.

Although there was no correlation between the P.C.V. and platelet count

changes, the patterns were similar on fasting, both reaching their highest levels between day 4 and 7. The mean rise in the platelet count at the follow up examination was not significant because there was great individual variation. The increase in some individuals may have been related to the stimulus of repeated blood loss (Wintrobe 1961).

Platelet adhesiveness does not appear to be increased in obesity. The mean adhesiveness during the basal period was $39.2 \pm 9.3\%$ which compares with a mean of $43.6 \pm 11.9\%$ in the group of 35 subjects who were chosen at random and who had their adhesiveness measured 20 minutes after venepuncture (Table IV).

The effect of fasting on platelet adhesiveness was very variable and overall there were no significant changes. Platelet adhesiveness increased in 9 and decreased in 9 cases. The pattern of change did not appear to be related to the changes in any of the other measurements made. In the case of the 2 subjects who undertook starvation on 2 occasions, the pattern of change was similar in one, while in the other although the basal level was the same, the adhesiveness rose markedly on one occasion and did not change in the other. Again there were no corresponding differences between the 2 fasts in any of the other measurements made in this subject.

Platelet adhesiveness at the follow up examination was not significantly different from that before starvation in spite of a significant fall in P.C.V. Weight loss therefore does not appear to influence platelet adhesiveness and also there was no correlation between platelet adhesiveness and the weight changes. Plasma NEFA levels were initially within the normal range although other workers (Opie & Walfish 1963, Glennon, Breck & Gordon 1965) have shown that the obese subjects have higher plasma NEFA levels compared to subjects of normal weight. In the basal period 16.1% of the apparent NEFA value obtained by single extraction was due to substances other than long chain fatty acids. During starvation this percentage rose to a maximum of 28% after 6 - 7 days due to the extraction of ketone bodies. The changes in plasma NEFA using the single extraction method paralleled those for the double extraction method, although basal and peak levels were higher. There was no correlation between plasma NEFA and age, degree of obesity or with

platelet adhesiveness. The basal plasma NEFA levels however correlated negatively with the basal blood sugar levels ($r = 0.52$ $p < 0.05$) which were normal in all cases. This correlation is compatible with Jenkins (1967) theory that in obesity, a state of increased insulin secretion occurs during which raised plasma NEFA levels and normal or low blood sugar levels are found. Later when pancreatic insufficiency arises fasting blood sugars tend to increase.

Despite the evidence that obese subjects do not mobilise fat as well as normal (Berkowitz 1964; Heald, Mueller & Daugela 1965; Opie & Walfish 1963 and Rabinowitz & Zierler 1962) only one subject, as assessed by the rise in plasma NEFA, did not mobilise fat adequately. This subject's plasma NEFA fell on fasting but nevertheless her platelet adhesiveness rose markedly (+31.2% by day 5 - 7).

It could be argued that the postulated effects of fatty acids on platelets might not become apparent until all the tight binding sites on albumin had been saturated (about 1212 $\mu\text{Eq/l}$), the reason being that only at above this level would fatty acids be free to adhere to cell walls. This level was exceeded in 8 out of the 16 subjects and yet no significant effect on the platelet count or platelet adhesiveness was apparent in this sub-group. In the 8 instances, in which fatty acids were doubly extracted, it must be conceded that the levels were generally lower. 1212 $\mu\text{Eq/l}$ was exceeded only twice but again there was no effect on platelets or clinical evidence of thrombosis in these subjects.

Starvation produced significant alteration of the plasma NEFA pattern which was maximal at day 4 - 5. The proportion of oleic acid was increased at the expense of palmitic, stearic and to a lesser degree myristic acid at this time. The pattern tended to return towards the baseline thereafter but refeeding resulted in a further significant increase in oleic and decrease in palmitic acid. By the time of the follow up examination, the pattern had returned to the baseline. The increase in the proportion of unsaturated fatty acids on starvation is the result of increased utilisation of NEFA for energy, combined with an increase in mobilisation.

This has resulted in a transition of the plasma NEFA pattern towards that of depot fat.(Table XVI.)

| Author | Origin of fatty acids | concentrations of fatty acids % | | | | | |
|-------------------------|-------------------------|---------------------------------|------------------|---------------------|-----------------|---------------|------------------|
| | | myristic 14:0 | palmitic 16:0 | palmitoleic 16:1 | stearic 18:0 | oleic 18:1 | linoleic 18:2 |
| present study | plasma NEFA before fast | 2.3 | 27.5 | 7.0 | 10.0 | 42.7 | 10.3 |
| | after 4 - 5 day fast | 2.2 | 24.1 | 6.4 | 7.9 | 48.4 | 10.9 |
| Insull & Bartsch (1967) | depot fat | 2.7 | 22.7 | 5.3 | 7.0 | 49.9 | 9.2 |

Table XVI Mean changes in serum NEFA pattern before and after 4 -5 day fast compared to levels in depot fat.

The fasting blood sugar in the basal period was, as already stated, within normal limits. There was no correlation with platelet adhesiveness during the basal period nor was there any correlation between the changes in platelet adhesiveness and in blood sugar. The blood sugar fell considerably on fasting, reaching the lowest level at day 4 - 5. It rose thereafter but remained significantly below the basal level for the first week of fasting. The fall in blood sugar during starvation is due both to carbohydrate withdrawal and to the hyperinsulinism which results from Ketosis (Madison, Mebane, Unger, & Lochner 1964).

After one day's fasting, 5 patients had significant Ketonuria while 4 had a trace. By the next day the majority of patients had moderate

Ketonuria and only 2 had negative Acetests. All but one had heavy Ketonuria by the 6th day and this persisted until the end of the fast. The Ketosis, like the fall in the blood sugar is due to carbohydrate deprivation (Azar & Bloom, 1963) which results in fat catabolism being required to supply the energy demands of the body. Changes in platelet adhesiveness did not correlate in any way with the development of Ketosis.

The effects noted on starvation were dominated by the fluid balance changes. The diuresis of starvation can be terminated by either carbohydrate (Wright, Gann & Albertson 1963) or protein feeding (Katz, Hollingsworth & Epstein, 1968). The cause of the diuresis is not understood. The fluid loss appears to be limited by secondary aldosteronism which appears to be mediated via the renin-angiotensin system (unpublished observations).

SUMMARY

There was no evidence of increased platelet adhesiveness in obesity a condition known to predispose to ischaemic heart disease. The changes in platelet adhesiveness following starvation were very variable but overall there did not appear to be any trend, the mean adhesiveness during starvation being close to the basal levels. The patterns of change in platelet adhesiveness were not related to the changes in plasma NEFA, blood sugar, Ketonuria, P.C.V. or serum cholesterol or triglyceride levels. Weight loss was not associated with any significant change in platelet adhesiveness.

Chapter 12

Effects of noradrenaline and isoprenaline administration

In all previously described experiments, the possibility that the alterations in platelet adhesiveness could have been related to catecholamine release is raised. It has also been suggested that the fatty acids and platelet changes could be functions of catecholamine release and therefore not be directly related to one another. In view of the known effects of adrenaline on platelets both in vivo (Besterman, Myat & Travadi 1967) and in vitro (O'Brien, 1963), it is important to know whether the changes in platelet adhesiveness are related to plasma NEFA directly or to adrenaline directly.

It was considered that the giving of adrenaline to normal subjects could be hazardous in view of its ability to produce ventricular fibrillation. Noradrenaline is known to have similar although less powerful effects on platelets but has little effect on the myocardium. The effects of noradrenaline infusion were therefore studied and its effects compared with those resulting from sublingual isoprenaline which is thought not to affect platelets (O'Brien, 1963).

The effects of noradrenaline and isoprenaline were studied in 6 fasting subjects. The noradrenaline group consisted of 5 males, and 1 female with an average age of 38.3 years, the isoprenaline group of 2 males and 4 females with an average age of 25.7 years. All were healthy at the time of experiment. Their diagnoses were as follows:-

| | |
|------------------------|---|
| attempted suicide..... | 2 |
| asthma..... | 2 |
| duodenal ulcer..... | 1 |
| Obesity..... | 1 |
| Parkinsonism..... | 1 |
| normal..... | 3 |

Both experiments were carried out on two of the males. The subjects remained horizontal lying on a bed throughout the experiment.

Noradrenaline Experiment

An intravenous saline infusion was set up and the subjects were given at least 20 minutes following this before the first blood sample was withdrawn. Two blood samples were withdrawn in this basal state. The infusion was then switched to saline containing noradrenaline in a concentration of 2 mg./litre. 100ml. of this solution (0.2mg. noradrenaline) was given over a period of 20 minutes following which 4 blood samples were taken at 5 minute intervals while the saline infusion was continued. Pulse rate and blood pressure were recorded at 5 minute intervals during the experiments. Platelet count, platelet adhesiveness, P.C.V. plasma NEFA and blood sugar were measured on each blood sample except in the one subject for whom there were no plasma NEFA or P.C.V. results.

Infusion of noradrenaline caused an elevation of the average platelet adhesiveness for the whole group persisting to the end of the experiment. This elevation was statistically significant only at 10 minutes (sample 5) after the noradrenaline. Platelet adhesiveness was increased immediately after the noradrenaline (sample 3) in 5 cases out of 6; at 5 minutes after (sample 4), in 3 out of 6; at 10 minutes after in 5 out of 6; and at 15 minutes after (sample 6) in 4 out of 6 cases. The average results are shown in table XVII.

The platelet count rose sharply in all cases after the noradrenaline infusion and this was statistically significant. It fell again quickly and 10 minutes after the infusion, the average value had almost reached the basal level.

The P.C.V. rose significantly immediately after the noradrenaline coinciding with the rise in platelet count. The rise in platelet count was greater than could be accounted for by the changes in P.C.V. The P.C.V. had also reached the basal level within 10 minutes of the end of the noradrenaline infusion.

The plasma NEFA levels increased in all cases at some time following the

| time | 0 min | 20 min | 40 min | 45 min | 50 min | 55 min |
|--------------------------------------|--------------|--------------|-----------------|----------------|----------------|--------------|
| sample number | 1 | 2 | 3 | 4 | 5 | 6 |
| time after infusion | | | 0 min | 5 min | 10 min | 15 min |
| platelet adhesiveness % | 31.9 ± 8.1 | 27.7 ± 7.7 | 34.5 ± 4.5 | 31.7 ± 13.2 | 34.5 ± 3.5 * | 35.8 ± 8.0 |
| platelet count x10 ³ /cmm | 146.1 ± 17.7 | 147.9 ± 17.6 | 192.1 ± 24.4 ** | 154.5 ± 38.2 | 149.6 ± 25.7 | 143.4 ± 23.8 |
| haemotocrit % | 45.5 ± 2.9 | 44.5 ± 2.0 | 48.1 ± 2.5 ** | 45.4 ± 2.2 | 44.8 ± 1.6 | 44.7 ± 1.4 |
| Plasma NEFA MEq/l. | 420.4 ± 102 | 405.8 ± 98 | 633.2 ± 244 | 736.8 ± 248 | 570.2 ± 116 | 538 ± 153 |
| blood sugar 1g/100 ml | 67.5 ± 3.6 | 71.8 ± 2.9 | 80.7 ± 2.8 *** | 86.3 ± 5.7 *** | 79.0 ± 5.7 *** | 72.8 ± 7.0 |

* p < 0.05
 ** p < 0.01
 *** p < 0.001

Table XVII
 Effect of noradrenaline infusion given in the 20 minute period between samples 2 & 3.

The results are the means (± S.D.) for 6 subjects

noradrenaline infusion. The changes were not statistically significant for any of the samples however because in one out of 5 cases at each sampling time, the plasma NEFA level was reduced below the mean basal level. When platelet adhesiveness was significantly elevated (sample 5) the plasma NEFA levels which had reached a peak in sample 4 were falling. There was no correlation between the changes in platelet adhesiveness and the changes in plasma NEFA.

Noradrenaline produced a significant rise in the blood sugar levels and this was maximal 5 minutes after the end of infusion (sample 4). Thereafter the blood sugar tended to fall and by the end of the experiment, 15 minutes after the noradrenaline had almost reached basal level.

The effect of the saline infusion can be judged from the changes during the basal period. Platelet adhesiveness, the P.C.V., plasma NEFA all tended to fall slightly while the blood sugar level rose and the platelet count remained unaltered. None of these changes was significant. The dose of noradrenaline was sufficient to lower the pulse rate and raise the blood pressure during the infusion in all cases. The effects on one subject (typical of the others) is shown in Figure 12.

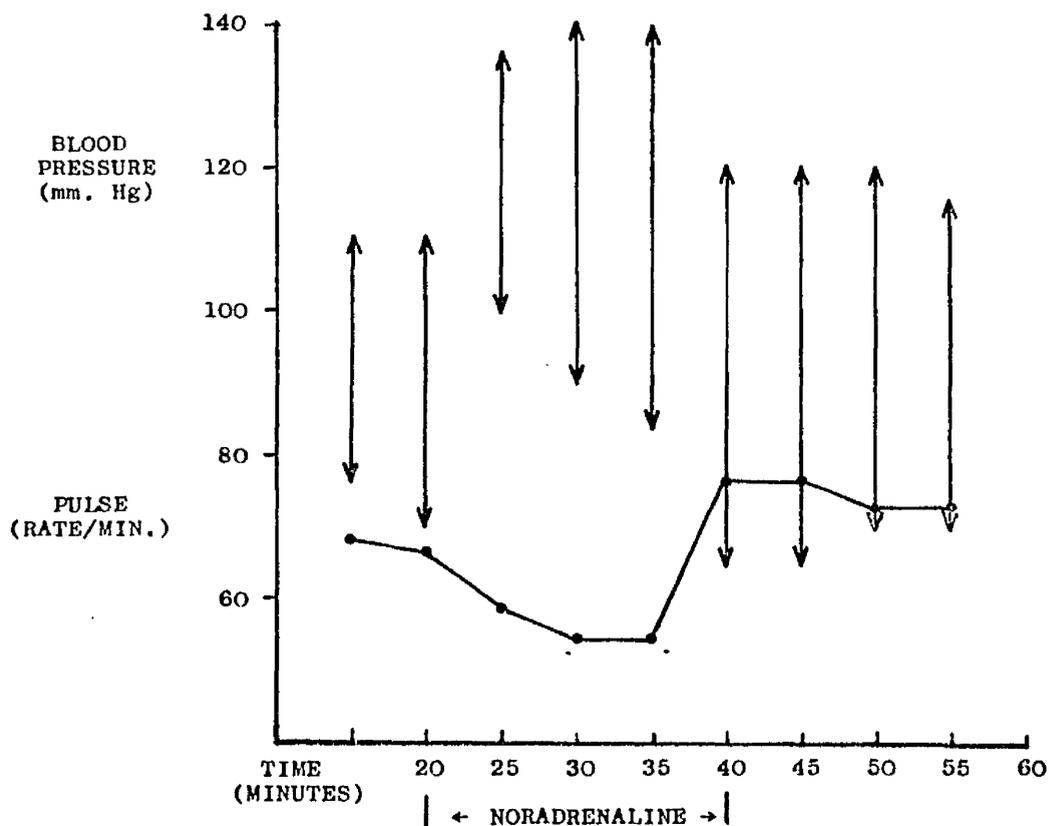


Figure 12.

Effects of noradrenaline infusion on the pulse and blood pressure of one subject.

These changes ceased almost immediately on stopping the noradrenaline, the blood pressure returning to the basal level while the pulse rate tended to 'overshoot' slightly, being very slightly above the basal level in the post infusion period.

Isoprenaline experiment

The subjects were given at least 20 minutes to reach basal conditions before the first of the two basal blood samples was withdrawn. 20 mg. isoprenaline was given sublingually during the 10 minutes following withdrawal of second basal blood sample. Blood samples were taken following administration of the isoprenaline and at ten minute intervals thereafter until 4 samples had been obtained. The interval between the samples was increased to 10 minutes compared with the 5 minutes for noradrenaline, because the effects of isoprenaline as assessed by its effect on the cardiovascular system were more prolonged.

The isoprenaline produced a tachycardia with a widening of the pulse pressure in all cases. The timing of the cardiovascular effects varied but in general commenced after the tablet had been completely dissolved.

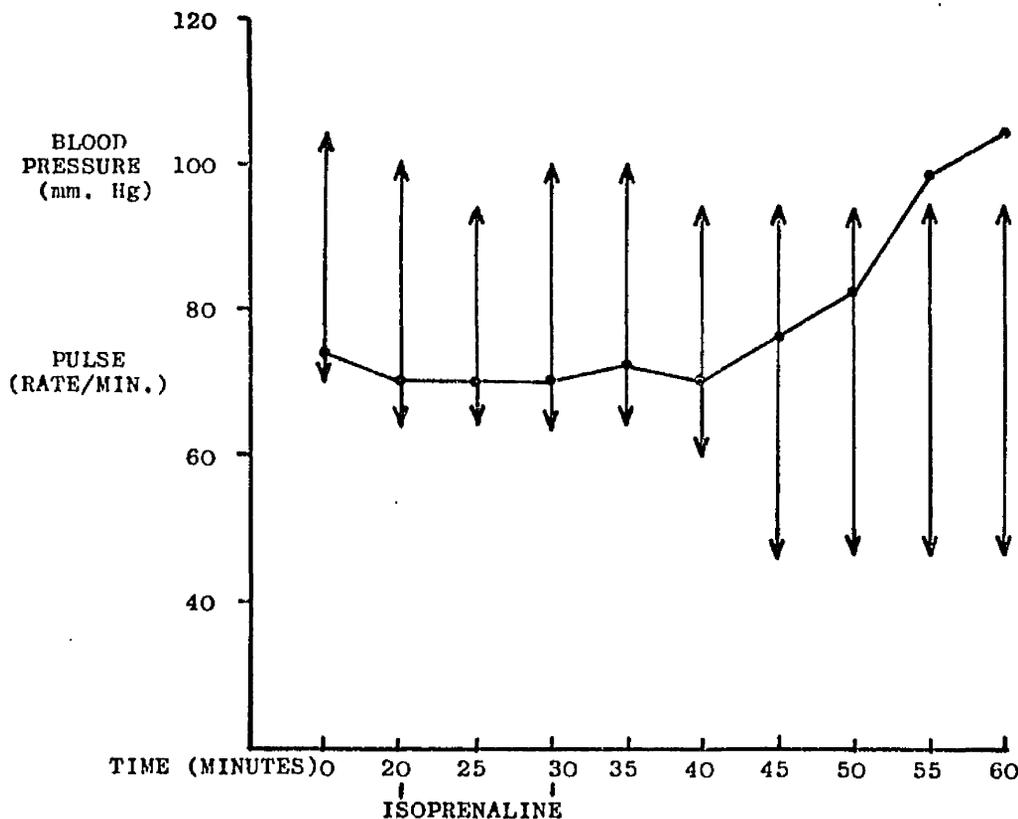


Figure 13.

Effects on the blood pressure and pulse rate of one subject of taking 20 mg. isoprenaline sublingually.

Fig.13 shows the effect on one of the subjects who was thought to have swallowed most of the dose. It appeared therefore that the rate of absorption varied from subject to subject, depending on the proportion of the dose which was absorbed sublingually. In general post isoprenaline blood samples were withdrawn while there were cardiovascular effects of the drug present.

Platelet adhesiveness, platelet count, plasma NEFA and blood sugar were measured in all blood samples. P.C.V. was not measured in 2 subjects. The result for the group is shown in Table XVIII. The results for platelet adhesiveness, platelet count, and P.C.V. have been presented with the mean basal level instead of the basal values separately because these measurements were not carried on one of the basal samples in one subject.

The most striking change in the platelets was a drop in the total count. This was detected immediately after the isoprenaline in 5 out of 6 instances. The change in platelet count was not statistically significant at this stage but it was significant 10 minutes (sample 4) ($p < 0.01$) and 20 minutes (sample 5) ($p < 0.05$) after the isoprenaline. The platelet count was still depressed in 5 out of 6 cases 20 minutes after the isoprenaline.

There were no corresponding changes in the P.C.V. Indeed when the platelet count was most significantly depressed, the P.C.V. was on average slightly elevated. None of the changes in P.C.V. was significant.

Platelet adhesiveness in spite of the fall in the total platelet count was elevated in every case at some period following isoprenaline. The increase was only statistically significant 20 minutes after isoprenaline (sample 5) being present in every case at that time. Thirty minutes (sample 6) after the isoprenaline, platelet adhesiveness was still elevated in 5 out of 6 instances, the sixth showing practically no change (-2.3%)

The changes in plasma NEFA were not striking. The highest individual rise was 208 $\mu\text{Eq./l.}$ which compares with the average rise of over 300 $\mu\text{Eq./l.}$ which followed noradrenaline. The plasma NEFA was elevated in every case at some point following isoprenaline the mean levels showing a slight non-significant rise.

There was slight but significant rise in the blood sugar level during

| times (mins) | 0 min | 20 min | 30 min | 40 min | 50 min | 60 min |
|--------------------------------------|-------------|-------------------|---------------|-----------------|----------------|--------------|
| sample number | 1 | 2 | 3 | 4 | 5 | 6 |
| time after isoprenaline | | | 0 min | 10 min | 20 min | 30 min |
| platelet adhesiveness % | | ⊕ 27.2 ± 5.0 | 29.7 ± 7.5 | 32.6 ± 9.9 | 34.8 ± 6.6 * | 40.4 ± 13.9 |
| platelet count x10 ³ /cmm | | ⊕ 196.3 ± 54.7 | 176.4 ± 63.4 | 169.1 ± 46.5 ** | 171.3 ± 35.1 * | 173.1 ± 45.1 |
| haematocrit % | | ⊕ 42.6 ± 5.2 | 42.9 ± 3.7 | 43.6 ± 4.5 | 41.8 ± 9.7 | 43.4 ± 4.2 |
| plasma NEFA μEq/l. | 488 ± 170 | 497 ± 166 | 514 ± 218 | 535 ± 244 | 468 ± 193 | 510 ± 206 |
| blood sugar mg/100ml. | 68.5 ± 12.7 | 72.5 ± 13.9 *** | 78.7 ± 17.6 * | 90.8 ± 22.0 * | 94.3 ± 24.0 ** | 87.7 ± 31.9 |

Table XVIII Effects of isoprenaline (20.m.g. sublingually) taken in the ten minutes between samples 2 and 3. The results are the mean(± S.D.) for 6 subjects.

⊕ mean basal level

* p < 0.05

** p < 0.01

*** p < 0.001

the basal period. It occurred in all cases. The changes following administration of isoprenaline were more marked than after noradrenaline. The maximum individual increment being 40mg/100ml. after isoprenaline compared with 20 mg./100ml. after noradrenaline. The average increments reflect this difference also, being 23.8 mg./100ml. against 16.6mg./100ml. The maximum increase in blood sugar occurred much later following isoprenaline.

Discussion

When Ahlquist(1948) classified the catecholamine drugs on the basis of their action, he found that the two ends of the spectrum were represented by noradrenaline and isoprenaline. Noradrenaline stimulated α receptors almost entirely, while isoprenaline stimulated β receptors. Adrenaline itself lies in the middle, stimulating both α and β receptors. Noradrenaline, acting on α receptors, has very little cardioaccelerator action and does not relax bronchial muscle. It does however constrict cutaneous and splanchnic vascular beds, resulting in an increase in the peripheral resistance producing an increase in the blood pressure and reflex cardiac slowing. Isoprenaline acting on β receptors, raises the heart rate and dilates the vascular beds in skeletal muscle leading to a lowering of the blood pressure and widening of the pulse pressure. Isoprenaline because of its β stimulation, dilates bronchial muscle and this is its main use therapeutically. These cardiovascular effects of noradrenaline and isoprenaline were noted in the present study. The effects of isoprenaline both on platelets and on the cardiovascular system were delayed compared to adrenaline. This is due simply to the difference in the route of administration.

The finding of a greater rise in plasma NEFA following noradrenaline than isoprenaline is in line with the observations of Mueller & Horwitz (1962) who used equipressor doses. It should be made clear at this point that the doses given in the present study were not equivalent on any basis. The dose of noradrenaline was approximately the same as that used by Mueller & Horwitz (1962) while the dose of isoprenaline was very much larger although of course given orally.

Having found a greater rise in plasma NEFA levels with noradrenaline compared to isoprenaline one would expect the blood sugar changes also to be similar to those of Mueller & Horwitz who found that isoprenaline did not alter the blood sugar. It was therefore a surprise to find that the blood sugar changes were greater with isoprenaline. The reason for this cannot adequately be explained. It may have been however, that some other unknown factor may have pushed up the blood sugar level in the present study as this is suggested by the significant rise during the basal period. There is a dispute in the literature (reviewed by Ellis 1956) concerning the relative ability of isoprenaline to raise blood sugar levels. It may be therefore that the present discrepancy with the results of Mueller & Horwitz is due to a difference in dosage but as pointed out already it is then surprising that the fatty acid responses were similar in proportion.

The two drugs produced markedly different effects on the platelet count. The rise following noradrenaline was similar to that usually seen following adrenaline. The rise was out of proportion to the P.C.V. changes and indicated an absolute increase in the platelet count. In rabbits, Sen, Muklerjee & Maiti (1953) have shown that the shortening of the clotting time correlates with the increase in the platelet count following adrenaline and that both changes can be prevented by exclusion of the spleen from the circulation. The increase in the platelet count might therefore be due to splenic contraction although whether the spleen is capable of this in man is not known. The fall in the platelet count following isoprenaline was a surprising finding. As there were no corresponding changes in the P.C.V, this would appear to represent a real fall in the platelet count. The most likely explanation of this fall is intravascular aggregation. Although noradrenaline is known to be able to aggregate platelets in vitro isoprenaline is thought to be inactive in this regard (O'Brien, 1963). In spite of this, the most probable explanation is that the isoprenaline aggregated the platelets in vivo. This has not previously been reported. An analagous situation might be the effect of a stimulator of α and β receptors in combination

with an α blocking drug since this would produce only B stimulation like isoprenaline. McClure, Ingram & Jones (1965) have done this using phentolamine and adrenaline. They found a 25% decrease in the platelet count following the infusion of phentolamine prior to the administration of adrenaline. Adrenaline then produced a slight further fall in the platelet count but the rise in platelet adhesiveness following adrenaline was not prevented. The present experiment is similar in many ways. Beta stimulation by isoprenaline produced a fall in the platelet count and platelet adhesiveness was increased despite the fall in the platelet count.

The increase in platelet adhesiveness following isoprenaline like the other effects on blood sugar, plasma NEFA and the cardiovascular system occurred later than after noradrenaline. It is difficult to compare the changes in adhesiveness because of the fall in the platelet count after isoprenaline. If one accepts that intravascular aggregation of platelets occurred and that these would be expected to be the most sticky platelets then the true rise in platelet adhesiveness following isoprenaline would be much greater than after noradrenaline. Since noradrenaline increased plasma NEFA levels more than isoprenaline, and isoprenaline increased blood sugar levels more than noradrenaline, it is clear that the positive correlation between plasma NEFA and platelet adhesiveness and the negative correlation between blood sugar and platelet adhesiveness suggested by the smoking experiment do not hold.

The most likely explanation of correlations between platelet adhesiveness and plasma NEFA and blood sugar noted in the smoking experiment is that all these changes have been results of catecholamine or nicotine stimulation. It seems most likely that isoprenaline, noradrenaline and other catecholamines act directly on platelets and not via plasma NEFA or blood sugar changes.

SECTION III

THE EFFECTS ON PLATELET ADHESIVENESS
OF THE ORAL ADMINISTRATION OF SUBSTANCES
KNOWN TO AFFECT LIPID METABOLISM

Chapter 13

Effect of administration of oral fat.

Patients with ischaemic heart disease have a more intense and more prolonged lipaemia than control subjects following a standard fat meal. This appears to be due to differences in the absorption of the fat from the alimentary tract since no difference between patients and controls can be demonstrated after fat given intravenously (Bouchier & Bronte-Stewart, 1961). Philip & Payling Wright (1965) have shown that platelet adhesiveness is elevated 2 hours after a fat meal and have attributed this to the lipaemia although they did not show any correlation between the two. They also speculated that fatty acids might be involved in producing the elevation in platelet adhesiveness. The opportunity to study platelet adhesiveness changes under conditions known to be abnormal in ischaemic heart disease - namely alimentary lipaemia - was therefore taken.

Method and Results

Seventeen patients were investigated during the lipaemia resulting from a liquid meal containing 75 G of milk fat (Nestles Rich Cream 325G; complan 35 G; glucose 15 G; water to 750ml.). The average age of the patients was 48.5 years (range 26 - 67 years) and there were 14 males and 3 females in the group. The diagnoses were as follows:-

| | |
|-------------------------------|---|
| ischaemic heart disease..... | 6 |
| peripheral vascular disease.. | 5 |
| normal..... | 2 |
| hernia..... | 1 |
| respiratory infection..... | 1 |
| asthma..... | 1 |
| congenital heart disease..... | 1 |

The patients were fasted overnight. Following the withdrawal of a basal

blood sample, the liquid fat meal was given. Blood samples were withdrawn at 1 hour, 2 hours, 4 hours, 6 hours and 8 hours thereafter. Throughout the experiment the patients remained in bed and were not allowed to smoke or eat. Platelet count, platelet adhesiveness, blood sugar, plasma NEFA, serum triglyceride and serum cholesterol were measured on all blood samples.

The average results are shown in Table XIX.

Blood sugar levels rose in all cases and were significantly elevated 1 hour, 2 hours and 4 hours after the fat meal. Even at 8 hours the mean level had not returned to the baseline, being elevated in 9 cases.

The plasma NEFA level fell initially, the depression being statistically significant at 2 hours. Following this however the levels became statistically elevated and remained so to the end of the experiments. The peak in the fatty acid curve was at 6 hours.

Serum triglyceride rose to a peak at 4 hours and then declined, almost reaching the baseline by 8 hours. The elevation was statistically significant at 1, 2, 4 and 6 hours.

Serum cholesterol also rose, the mean level being at its highest at 2 hours. The elevation was statistically significant at all times after the fat meal.

The platelet count showed only minor insignificant fluctuations.

Platelet adhesiveness was raised above the basal level at 2 hours in 12 out of 17 instances and this change was significant at the 5% level. None of the changes at the other times was significant. There appeared to be basically 2 types of response with regard to platelet adhesiveness. In one group, after the initial elevation at 2 hours, platelet adhesiveness was depressed at 4 hours returning to the basal level at 6 and 8 hours. In the other group, platelet adhesiveness continued to rise to a peak at 4 hours thereafter declining. The changes in the 2 groups, consisting of 9 and 8 individuals each, are shown in Figure 14. The groups did not differ in age, sex or diagnostic composition. The first group had initially higher platelet adhesiveness levels but this difference was not significant. The blood sugar and plasma NEFA responses of the groups were similar but the second group tended to have a higher and more prolonged lipaemia as measured

| | fasting | 1 hour | 2 hours | 4 hours | 6 hours | 8 hours |
|---|--------------|------------------|------------------|-------------------|-------------------|-------------------|
| platelet adhesiveness % | 40.9 ± 10.8 | 42.1 ± 12.7 | 47.4 ± 11.2 * | 42.4 ± 19.2 | 47.1 ± 17.3 | 42.6 ± 10.1 |
| platelet count x10 ³ /cmm. | 177.5 ± 57.4 | 179.6 ± 69.6 | 174.9 ± 52.0 | 173.3 ± 54.5 | 183.0 ± 51.5 | 172.2 ± 58.3 |
| non adhesive count x 10 ³ /cmm. | 106.6 ± 43.9 | 107.7 ± 53.0 | 92.3 ± 33.1 * | 100.0 ± 56.9 | 95.2 ± 37.3 | 96.4 ± 27.9 |
| blood sugar mg/100ml | 66.7 ± 11.3 | 76.5 ± 15.5 *** | 74.8 ± 13.3 * | 77.4 ± 14.2 *** | 73.7 ± 12.1 | 70.8 ± 9.5 |
| plasma NEFA MEq./l. | 425.9 ± 98.3 | 381.5 ± 136.7 | 362.4 ± 99.9 * | 530.1 ± 111.8 *** | 646.1 ± 179.0 *** | 597.2 ± 139.9 *** |
| serum triglyceride mg/100ml | 106.9 ± 22.4 | 116.9 ± 31.4 ** | 161.5 ± 46.6 *** | 205.6 ± 82.5 *** | 179.6 ± 82.8 *** | 124.6 ± 56.4 |
| serum cholesterol mg/100ml | 246.4 ± 44.8 | 257.6 ± 51.0 *** | 262.9 ± 59.9 ** | 260.0 ± 53.8 ** | 257.3 ± 57.4 * | 259.2 ± 56.5 * |

Table XIX Results (means ± S.D.) for 17 fat tolerance tests

* p < 0.05
 ** p < 0.01
 *** p < 0.001

by the serum triglyceride. The differences between the groups were not significant in any respect and it should be emphasised that the groups were formed merely on the basis of whether platelet adhesiveness was elevated or depressed at 4 hours.

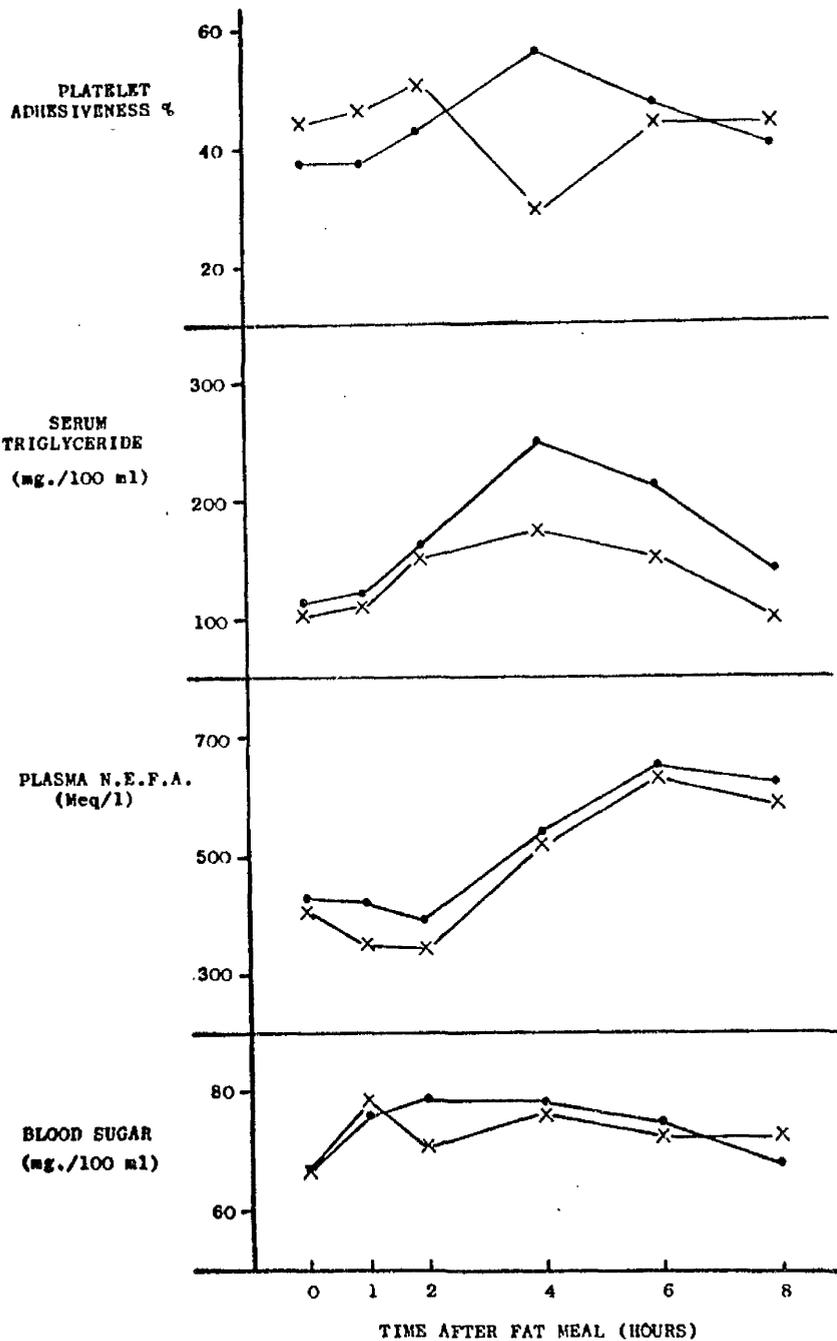


Figure 14.

Mean results for platelet adhesiveness, serum triglyceride, plasma NEFA and blood sugar for 9 subjects whose platelet adhesiveness was depressed 2 hours after a fatty meal (X-X) compared to those for 8 subjects in whom platelet adhesiveness was increased 2 hours after a fatty meal (·-·).

Discussion

The present results confirm the finding of Philip and Payling Wright (1965) of elevation of platelet adhesiveness 2 hours after a fatty meal. Blood sugar, serum cholesterol, serum triglyceride are significantly elevated at this time while plasma NEFA is significantly depressed. The platelet changes following the meal were irregular and basically followed the two patterns already described. The changes in platelet adhesiveness did not follow or correlate with the changes in any of the other measurements. If the lipaemia was responsible for the platelet changes, then one would have expected that platelet adhesiveness would have reached a peak at 4 hours or later. This did not happen. The suggestion made by Philip & Payling Wright that increase in the plasma NEFA levels might be responsible also would appear unlikely since plasma NEFA levels were depressed at this time. In view of the work of Bridges, Dalby, Millar & Weaver (1965) showing that platelet adhesiveness is elevated following glucose alone, it is interesting to note that the blood sugar levels were also elevated at 2 hours. Here again, the platelet adhesiveness was not elevated at 1 hour and 4 hours, when the blood sugar was raised.

It was decided to investigate the effect of oral glucose on platelet adhesiveness because the reported elevation of platelet adhesiveness, following glucose had been denied by Bennett (1967) using Hellem's & Borchgrevink's methods and because the effect of glucose could be responsible for the rise in platelet adhesiveness after the fat meal in the present experiment.

Chapter 14Effects of oral glucose

Ten subjects were investigated. The group consisted of 5 males and 5 females with an overall average age of 57.4 years. The diagnoses were:-

| | |
|------------------------------|---|
| diabetes..... | 3 |
| asthma..... | 2 |
| normal..... | 2 |
| ischaemic heart disease..... | 2 |
| peripheral vascular disease. | 1 |

These subjects were fasted overnight and drank 50G of glucose in water after a basal blood sample had been taken. Blood samples were then taken half hourly for 2½ hours. Throughout this period the subjects remained in bed and were not allowed to eat, drink or smoke. Platelet count, platelet adhesiveness, blood sugar and plasma NEFA levels were measured on each sample.

The mean results for the 10 subjects are shown in Table XX.

The blood sugar results give a mean curve which is 'diabetic'. There were only 3 diabetics in the group however. The very high levels in two of these (fasting - 235mg./100ml. which a peak of 552mg./100ml. and fasting - 155mg./100ml. with a peak of 251mg./100ml.) has produced this effect. Of the remaining 'normal' curves, 3 had a peak at 30 minutes and 4 at a peak of 60 minutes.

The plasma NEFA levels of the diabetics were not higher than the others. Glucose produced suppression of plasma NEFA in all cases. The lowest average for plasma NEFA levels occurred at 1½ hours, but in 2 normals and 1 diabetic the lowest level had been reached by 1 hour. The mean depression of the plasma NEFA was significant at 1, 1½ and 2½ hours.

Mean platelet adhesiveness rose progressively following the administration of glucose. The elevation became significant at 1½ hours and at 2½ hours.

| time | 0 | ½ hr | 1 hr. | 1½ hr. | 2 hr. | 2½ hr. |
|---|---------------|-----------------|----------------|-----------------|---------------|----------------|
| platelet count x10 ³ /cmm | 263.4 ± 46.9 | 270.1 ± 52.5 | 256.4 ± 47.0 | 260.5 ± 52.2 | 250.2 ± 48.1 | 261.1 ± 39.5 |
| platelet adhesiveness % | 27.3 ± 7.8 | 28.9 ± 9.7 | 30.7 ± 4.7 | 32.7 ± 8.3.* | 34.7 ± 14.7 | 39.1 ± 15.3* |
| blood sugar mg/100ml. | 97.0 ± 55.3 | 161.9 ± 80.4*** | 187.6 ± 139.6* | 163.6 ± 109.5** | 139.7 ± 115.0 | 121.1 ± 110.9 |
| plasma NEFA MEq./l. | 440.0 ± 184.4 | 408.0 ± 124.5 | 266.0 ± 70.4** | 235.6 ± 64.9** | 316.5 ± 183.6 | 319.2 ± 138.9* |

TABLE XX
Mean results (± S.D.) for 10 subjects given
50G of glucose orally.

* p < 0.05
** p < 0.01
*** p < 0.001

The increase at 2 hours just failed to be statistically significant. At this time however, the initial platelet count had fallen in 9 out of the 10 instances and this probably explains the discrepancy. The small changes in the initial platelet count were not significant despite the fall in 9 out of 10 instances at 2 hours. The rise in platelet adhesiveness was in general later than that of the blood sugar, the peak level being recorded at 2½ hours, in 6, 2 hours in 1, 1½ hours in 2 and ½ hour in 1. There was no correlation between these changes or between those of platelet adhesiveness and plasma NEFA.

Discussion

The finding of a rise in platelet adhesiveness after ingestion of glucose confirms the work of Bridges, Dalby, Millar & Weaver (1965) who used basically the same method. The results differ in the timing however. Bridges et al. found that the peak of the glucose curve generally corresponded to the maximal adhesiveness but that towards the end of the experiment (2 hours in their case) adhesiveness was tending to become less when the blood sugar levels particularly in the diabetics, were still considerably raised. Platelet adhesiveness in the present series tended to rise later than the blood sugar. The reason for this discrepancy is not clear but it probably indicates that the blood sugar changes are not responsible for the changes in platelet adhesiveness. This impression is borne out by considering the one subject who did not show an increase in platelet adhesiveness following glucose. This subject increased her blood sugar from a fasting level of 77mg./100ml. to a peak level of 202mg./100ml. at 1 hour, this being the greatest increase in blood sugar in the non-diabetic group. The increase in platelet adhesiveness following glucose may be only demonstrable using the Payling Wright method since Bennett (1967 b) was unable to find any change using Borchgrevink's 'in vivo' method or using the method of Hellem. An alternative explanation however would be that the discrepancy is due to timing. This appears the more likely explanation. Bennett compared only basal platelet adhesiveness with that 'approx. 1 hour. after the ingestion of 50G glucose'. The present results show a rise in platelet adhesiveness

subsequent to 1 hour and like Bennett's study, show no significant change in platelet adhesiveness at 1 hour.

The fall in the initial platelet count in 9 out of 10 cases at 2 hours probably explains why the increase in adhesiveness just failed to be statistically significant at that time. The fall in the platelet count because of its transient nature is likely to have been due to the loss of platelets in the process of withdrawal of the blood rather than due to intravascular aggregation. This fall in the platelet count is consistent with the increase in platelet adhesiveness since one would expect that loss of platelets would be more likely to occur when adhesiveness is increased.

There was no correlation between the changes in plasma NEFA and those of platelet adhesiveness. The lack of correlation is best seen again in the subject whose platelet adhesiveness did not alter. She had the highest initial plasma NEFA level (917 μ Eq./l.) and showed the greatest reduction (to 279 μ Eq./l.) The fact that this subject had the highest rise in blood sugar and the greatest fall in plasma NEFA and yet did not alter her platelet adhesiveness is completely contrary to the expected effects if one believed that the relationships demonstrated in the smoking experiments were valid.

The findings of increased platelet adhesiveness at approximately 2 hours after glucose and after fat, with no obvious relationship to either glucose or lipid levels, suggest that the phenomena may be due to a common mechanism. The reason for this increase is not clear but the implication that platelet adhesiveness should only be measured in the fasting state when comparing one subject with another is important.

Nobody, as far as I am aware, has reported any other evidence of hypercoagulability after the administration of oral glucose. Indeed, Isapogas, Cotton, Flute & Murray (1962) found increased fibrinolysis following the ingestion of 50G of glucose. The situation is quite different with regard to oral fat. Fullerton, Davie & Anastasopoulos (1953) have shown that the clotting time in non-water-wettable test tubes was shortened during the lipaemia following oral fat. Chylomicrons have been shown to play an important part in blood coagulation (Poole 1955b) in vitro

but Husom (1961) has suggested that the effects may be due to the addition of platelets along with chylomicrons. Swank (1959) has demonstrated an increase in blood viscosity and red cell aggregation following fat feeding in dogs. This occurred later than the peak of the lipaemia. He also demonstrated accompanying changes in the platelet count and in the clotting time. In the presence of red cell aggregation, the likeliest explanation of the fall in the platelet count is intravascular aggregation. The present study does not suggest that there is any platelet aggregation or increase in adhesiveness after the peak of the lipaemia. It is interesting that Begg & Hearn (1967) using a fat meal identical to the present one, were unable to demonstrate any change in blood viscosity in a group of 24 subjects, containing 12 with ischaemic heart disease, again suggesting that there is no significant red cell aggregation in man at this time. Intravascular agglutination and plugging in the bulbar conjunctiva following fat feeding has been seen by Williams, Higginbotham & Knisely (1957) in men with ischaemic heart disease while in normal subjects, there was little or no agglutination. Of the 11 subjects with evidence of vascular disease in the present study 7 showed slight increases in platelet count. There is thus no evidence of platelet aggregation. Philip & Payling Wright (1965) however did find a small decrease in the platelet count 2-2½ hours following oral fat and they speculated that this may have been due to intravascular aggregation. The present study agrees with their finding of increased adhesiveness at this time. Besterman et al. however did not find any alteration in the platelet count in their study. Their results have not been presented in detail, only showing the changes in adhesiveness and triglyceride in 2 out of 40 fat tolerance tests. From these tests, the increase in adhesiveness appeared to have been present between 3 and 7 hours, but did not appear to be related to the degree of lipaemia. In view of all these conflicting patterns following oral fat, it would appear that the most obvious explanation is that different types of meals have different effects. It is interesting that with regard to clotting at least several groups of workers are agreed that the degree of saturation of the ingested fats does not affect the results (Merskey & Nossel 1957), O'Brien (1957),

McDonald & Fullerton (1958).

Summary

Ingestion of a glucose load or a fatty meal is followed by an increase in platelet adhesiveness. This increase in adhesiveness does not appear to be related either to the blood sugar or serum lipid changes.

Chapter 15

Effect of dietary sucrose on serum lipids and platelet adhesiveness

Since ingestion of glucose is followed by an increase in platelet adhesiveness, one might expect sucrose to have a similar effect. Yudkin & Morland (1967) have shown that subjects who have had a myocardial infarction consume more sucrose than the general population. They have suggested on the basis of this, that sucrose may be an aetiological factor in ischaemic heart disease. It was therefore decided to investigate the effect of sucrose on a long term basis on platelet adhesiveness to determine if platelet adhesiveness might be the link relating sucrose to ischaemic heart disease. Could in fact the increased adhesiveness found in subjects with ischaemic heart disease be due to increased sucrose intake?

Another possibility might be that sucrose is related to ischaemic heart disease via serum lipids since Macdonald (1964, 1965 & 1967) has shown that these are elevated by sucrose compared to starch. Serum lipids were therefore measured not only to determine the effect of sucrose on them but in addition to determine if there was any relationship between the serum lipid and platelet adhesiveness changes.

Five subjects, 3 males and 2 females, were investigated. Their ages ranged from 49 to 85 with a mean of 63 years. Their diagnoses were:-

ischaemic heart disease.....2
 cerebrovascular accident.....1
 Parkinson's disease.....2

Following admission to hospital, the subjects ate the ordinary ward diet for 2 weeks. Their calorie requirements were assessed during this period. During the subsequent experimental period of 8 weeks, the calorie intake was kept constant. During one 4 week period, 70% of the carbohydrate was supplied as sucrose while during the other 4 week period, although the proportions of carbohydrate, protein and fat were not altered, sucrose was not given.

Throughout the whole period, 40% of the calories were given as fat, 15% as protein and 45% as carbohydrate. The sucrose free diet was given first in three instances. The effect of an isocaloric interchange between sucrose and starch could thus be determined. Blood samples were taken from all subjects thrice weekly after an overnight fast. Serum cholesterol, serum triglyceride, plasma NEFA, blood sugar, platelet count and platelet adhesiveness were determined in each sample.

The average results for the 5 subjects for each period are shown in Table XXI. The average results for each period for each of the 5 individuals is shown in Table XXII. Taking the overall results first, it can be seen that the only measure which changed significantly was the fasting blood sugar. This was higher on the diet containing sucrose. Considering each individual, this change in the blood sugar was significant in 2 and just failed to be significant in other 2 ($t = 2.042$ (for $p < 0.05$, $t = 2.109$) and $t = 2.082$ (for $p < 0.05$, $t = 2.093$)).

Plasma NEFA, serum cholesterol and serum triglyceride did not vary significantly overall or for any individual between the 'sucrose free' and sucrose periods.

Platelet count was higher in all cases when on the sucrose diet. The variation was significant for only one individual (L).

There was no change in platelet adhesiveness between the two periods. One individual (McI) however did show a significant change, the adhesiveness being significantly greater when on the 'sucrose' diet. The other subjects showed small insignificant changes and the overall means for each period were very close together.

Discussion

The mean platelet count was higher in all 5 individuals when they were on the diet containing sucrose. This was significant however in only 1 case (L). The reason for this increase is not clear. Blood volume changes might be involved. Alternatively one might expect an increase in platelet count if the platelets were less sticky and therefore less liable to aggregation and deposition both on endothelium and apparatus. This cannot be the

| | Without Sucrose | With Sucrose | t | Significance |
|----------------------------|--------------------|-------------------|-------|--------------|
| cholesterol mg% | 238.1 \pm 37.4 | 243.2 \pm 43.8 | 0.837 | N.S. |
| triglyceride mg% | 106.6 \pm 46.2 | 108.1 \pm 47.5 | 0.962 | N.S. |
| NEFA Meq/l. | 435.9 \pm 125.9 | 420.1 \pm 130.8 | 0.061 | N.S. |
| Blood glucose mg% | 65.8 \pm 8.1 | 72.2 \pm 6.6 | 4.300 | p < 0.001 |
| platelet adhesiveness % | 34.4 \pm 9.0 | 34.1 \pm 11.3 | 0.131 | N.S. |
| Weight Kg | 58.3 \pm 9.0 | 58.4 \pm 9.1 | 0.025 | N.S. |

TABLE XXI

Average results (with standard deviations) for the 5 subjects showing the effects of the isocaloric substitution of starch by sucrose.

| Subject | sucrose | McI | W | J | D | L |
|---|---------|--------------|--------------|--------------|--------------|--------------|
| platelet count (x10 ³ /cmm) | - | 156.7 ± 17.5 | 251.7 ± 31.1 | 225.7 ± 28.0 | 206.0 ± 21.7 | 192.4 ± 14.2 |
| | + | 163.3 ± 13.8 | 261.4 ± 22.4 | 230.4 ± 25.6 | 219.1 ± 25.8 | 229.0 ± 34.5 |
| platelet adhesiveness % | - | 40.4 ± 5.1* | 33.3 ± 8.8 | 36.8 ± 7.1 | 31.7 ± 8.7 | 31.3 ± 10.5 |
| | + | 47.3 ± 8.0 | 33.1 ± 9.9 | 37.4 ± 7.7 | 28.7 ± 7.1 | 26.3 ± 6.4 |
| blood sugar mg/100ml. | - | 67.9 ± 5.2* | 65.4 ± 11.6* | 65.2 ± 11.8 | 66.2 ± 6.4 | 64.8 ± 7.6 |
| | + | 74.3 ± 4.0 | 74.6 ± 3.5 | 74.0 ± 5.5 | 72.7 ± 7.9 | 66.3 ± 3.7 |
| plasma NEFA MEq/l. | - | 568 ± 80 | 382 ± 126 | 386 ± 92 | 450 ± 120 | 417 ± 89 |
| | + | 611 ± 98 | 355 ± 100 | 315 ± 65 | 474 ± 88 | 387 ± 84 |
| serum cholesterol mg/100ml | - | 267 ± 35 | 180 ± 11 | 222 ± 11 | 249 ± 18 | 279 ± 12 |
| | + | 270 ± 22 | 181 ± 15 | 220 ± 11 | 263 ± 23 | 275 ± 42 |
| serum triglyceride mg/100ml | - | 67 ± 14 | 69 ± 7 | 108 ± 12 | 187 ± 28 | 113 ± 13 |
| | + | 70 ± 7 | 73 ± 13 | 100 ± 13 | 188 ± 28 | 108 ± 23 |

TABLE XXII Mean results (± S.D.) for each measurement for each of the 5 subjects on the sucrose diet (+) and on the sucrose free diet (-).

Difference significant at p < 0.05 - *
p < 0.01 - **

explanation however since considering the results overall there was no change in platelet adhesiveness between the two periods. Indeed one subject showed a significant increase in platelet adhesiveness when on sucrose and yet the increase in platelet count was still apparent. Why did the adhesiveness increase in one case (McI) and not in the others? Again the reason is not clear. It is interesting that in this subject, the fasting blood sugar was significantly higher when on sucrose. In view of the work of Bridges et al. relating platelet adhesiveness to blood sugar levels it would be tempting to try and relate the two changes. Unfortunately this relationship does not hold since all the subjects showed small increases in the fasting blood sugar (this being significant in one other and just failing to reach significance in the other two) with if anything a tendency for platelet adhesiveness to fall slightly in these cases.

The present study fails to support the finding of Macdonald and his coworkers (1964, 1965 & 1967) that the isocaloric interchange of sucrose with starch produced lowering of the serum triglyceride levels. Kuo & Bassett (1965) had supported this view by finding that replacement of starch by sucrose raised serum triglycerides. Rifkind, Lawson & Gale (1966) also found that triglyceride levels were lowered by sucrose-free diet. Careful examination of these studies suggests that weight changes may have been involved in producing the lipid changes, so that in fact the 'sucrose free' diets were weight reducing diets. This view was put forward with the preliminary results of this study (Dunnigan, Fyfe & Sawers 1967). The failure of sucrose to have any extraordinary action on serum lipids has also been found by Lees (1965); Farquhar, Frank, Gross, Reaven (1966); Anderson (1967) and Antonis (1967). The findings of these workers agree with the present results and it would appear likely that sucrose does not have any lipid-raising property out of proportion to that of other carbohydrates. Doubt is present concerning the place of sucrose in the aetiology of ischaemic heart disease. The findings of Yudkin and his coworkers (1964 & 1967) that subjects with ischaemic heart disease take more sucrose than controls has been denied by Little, Shanoff, Csima, Redmond & Yano (1965) and also by Papp, Padilla & Johnson (1965).

Yudkin has suggested that the discrepancy between his own work and that of Little et al. may be due to the time interval between the study and the patient's myocardial infarction. To support this, he has shown that his patients reduce their sucrose intake voluntarily after their attack so that by 18 months after the attack their sucrose intake is not greater than that of controls. The study of Papp et al. was conducted in 18 out of 20 instances within 6 months of the subject's myocardial infarction and so this criticism does not hold in this case. Support for Yudkin's ideas comes from the work of Osancova, Hejda and Zvolankova (1965) and of Cohen, Bavly & Poznanski (1961). Osancova et al. have shown a positive correlation between the mortality from cardiovascular disease and the sugar consumption ($r = 0.7672$, $p = 0.01$) Cohn et al. have found that Yemenites resident in Israel have a much higher sugar consumption than those staying in the Yemen and also a higher incidence of ischaemic heart disease. The place of sucrose in the aetiology of ischaemic heart disease is therefore uncertain. Certainly there is growing evidence that it does not raise serum lipids out of proportion to other carbohydrates. It may on the other hand cause a relative impairment of glucose tolerance.

The finding that fasting blood sugars were greater on a diet containing sucrose is extremely interesting. Relative impairment of glucose tolerance while on sucrose compared to starch has been demonstrated by Cohen, Teitelbaum, Balogh & Groen (1966). The present finding also suggests that glucose tolerance may be relatively impaired on a sucrose diet compared to a sucrose free diet. This would seem a reasonable deduction since Frethem (1963) has shown that fasting blood sugar levels can be taken as an index of carbohydrate tolerance.

Further investigation into this suggestion of impaired glucose tolerance was carried out. Essentially the same experiment was carried out in a further 8 subjects but serum insulin levels were measured in addition to blood sugars and glucose tolerance tests were done once weekly. Table XXIII shows the results of this experiment.

There was a significant difference between the fasting blood sugars on the sucrose compared to the sucrose free diet. This was significant individually in 3 out of the 8 cases making a total of 5 out of 13 when

| time after 50G glucose | BLOOD SUGAR (mg/100ml.) | | P. | SERUM INSULIN (M units/ml) | | P. |
|------------------------------|-------------------------------|----------------------------|-------|-------------------------------|----------------------------|------|
| | without dietary sucrose | with dietary sucrose | | without dietary sucrose | with dietary sucrose | |
| fasting | 72.3 \pm 7.4 | 76.0 \pm 11.0 | <0.01 | 28.3 \pm 13.5 | 27.9 \pm 12.3 | N.S. |
| $\frac{1}{2}$ hr. | 110.2 \pm 16.7. | 117.5 \pm 7.4 | N.S. | 116.0 \pm 61.2 | 125.4 \pm 56.7 | N.S. |
| 1 hr. | 116.6 \pm 10.6. | 121.5 \pm 15.6 | N.S. | 132.2 \pm 75.8 | 135.5 \pm 72.8 | N.S. |
| 1 $\frac{1}{2}$ hrs. | 99.9 \pm 32.2 | 104.3 \pm 40.5 | N.S. | 96.1 \pm 42.7 | 98.0 \pm 65.8 | N.S. |
| 2 hrs. | 84.3 \pm 35.4 | 85.9 \pm 37.9 | N.S. | 62.2 \pm 33.8 | 62.4 \pm 41.1 | N.S. |

TABLE XXIII Mean (\pm S.D.) blood sugar and serum insulin results for 8 subjects during the sucrose and sucrose free dietary periods. Each subject had 4 glucose tolerance tests at weekly intervals during each dietary period.

the original 5 cases already described were added. Although the mean blood sugar values were slightly higher at each point during the glucose tolerance test there was no significant difference at any time or when the areas under the curves were compared. There was no difference between the fasting serum insulin concentrations on the 2 diets nor did the serum insulin levels become significantly different between the diets at any time during the glucose tolerance tests although the mean levels, like those for the blood sugars were higher at each point during the glucose tolerance test during the sucrose period. This suggests that the increase in fasting blood sugar during the sucrose diet might be due to increased insulin resistance. The elevation of the fasting blood sugar associated with unchanged fasting serum insulin levels, and also the greater mean blood sugar and serum insulin responses to glucose, during the sucrose period would fit into this concept. Glucose tolerance tests were only done once weekly compared to thrice weekly for the fasting specimens, and this may account for the failure of the difference between the glucose tolerance curves between the dietary periods to be statistically significant. In spite of this failure it is striking that the mean blood sugar and serum insulin responses were greater at every point during the glucose tolerance test during the sucrose diet.

It is surprising if the increased fasting blood sugar levels during the sucrose diet do represent impaired carbohydrate tolerance, that the differences between the glucose tolerance tests are not more striking since one normally uses a glucose tolerance test to demonstrate impaired carbohydrate tolerance rather than fasting blood sugars. A reason for this could be that a physiological balance has been struck between limiting the blood sugar response to oral glucose and increasing the insulin response. If either the blood sugar response or the serum insulin response to glucose had been fixed between the two dietary periods then the change in the other might have been significant.

A further possibility is that the insulin mechanism might not be involved in causing elevation of the fasting blood sugar. At no time were there significant differences in the plasma insulin levels between the two

dietary periods nor were the glucose levels during the glucose tolerance tests significantly different. It could therefore be argued that there is no evidence of impaired carbohydrate tolerance or increased insulin resistance depending on how one interprets the small non significant differences in serum insulin and blood sugar following glucose.

Cohen et al. (1966) found impaired glucose tolerance when on a sucrose diet but those workers did not measure serum insulin levels. The cause of the probable increase in insulin resistance is not clear. The present study has shown that it is not due to alteration of plasma NEFA levels which are known to affect insulin resistance nor is it associated with any change in serum cholesterol or serum triglyceride. The most likely explanation is that it is a direct effect of sucrose on carbohydrate metabolism.

Summary

Sucrose in the diet, which is thought by some to be a factor predisposing to ischaemic heart disease, was not associated with increased platelet adhesiveness. Fasting blood sugars were significantly higher when on a sucrose diet while fasting serum insulins were unchanged. The blood sugar and serum insulin responses to glucose tended to be greater when on the sucrose diet. Increased insulin resistance therefore appeared to be present during the sucrose diet but the cause for this is not apparent.

Chapter 16

Effects of γ -linolenic acid

The effect of mobilisation of endogenous fatty acids on platelet adhesiveness has been studied in some detail in Section 2 of this thesis. The next question to be considered - and this could have therapeutic application - is whether exogenous fatty acids given orally affect platelet adhesiveness. Stimulus to this line of research was given by Owren's group when they demonstrated that linseed oil appeared to lower platelet adhesiveness. Although this work has now been withdrawn, the in vitro studies of Kerr, Pirrie, MacAulay & Bronte-Stewart (1965) and of Mahadevan, Singh & Lundberg (1966) show that linolenic acid appears to protect platelets from aggregation by other fatty acids. A preparation containing γ -linolenic acid was made available by the Calmic Co. for study. This was the preparation "Calmic 131A" which is obtained from the seeds of evening primrose (*Oenothera lamarkian*). The composition of this preparation is:-

| | |
|--------------------------|------|
| palmitic acid | 6.5% |
| stearic acid | 1.5% |
| oleic acid | 10% |
| linoleic acid | 73% |
| γ -linolenic acid | 9% |

This drug therefore contains predominantly unsaturated fatty acids and might be expected to lower serum lipid levels in addition to any direct effect it might have on platelets. The effects of this preparation on platelet adhesiveness, plasma NEFA, serum cholesterol, serum triglyceride and blood sugar were therefore studied in 6 subjects. The average age of the group was 53 years (range 40 - 67). All were males and all had ischaemic heart disease except for one subject who was healthy. The investigation was carried out on an outpatient basis. The subjects were seen on three occasions before commencing Calmic 131A. They then

commenced the drug in a dose of 3 capsules (825 mg) a day for 2 weeks. They were seen again on 3 occasions during the second week of treatment. On each occasion, the subjects were seen at 9 a.m. in the fasting state. Blood was withdrawn for the platelet and lipid studies on each occasion.

Results

Table XXIV shows the mean results (\pm S.D.) for the group as a whole before and during Calmic 131A administration. The only change which was significant was in serum cholesterol, which fell. The means for plasma NEFA, serum triglyceride, blood sugar and platelet count were virtually unchanged. Platelet adhesiveness did not change significantly but tended to fall. Table XXV shows all the platelet adhesiveness results for each individual together with the means for both periods for each subjects.

It can be seen from these results that platelet adhesiveness was varying spontaneously during both periods. Although the mean value for adhesiveness while on Calmic 131A was lower in the majority of subjects, this cannot be attributed to the drug because the degree of variation within each period was greater than the variation between the periods. For example, for subject 6, platelet adhesiveness varied from 25.4 to 51.8 during the basal period and from 30.4 to 46.4 while on Calmic 131A, with a difference between the means for each period of only 2.7%. The overall changes were similar. The difference between the means for samples (bottom line Table XXV) during the basal period was 4%, during treatment was 4.4% and between the basal and treatment periods only 4.1%.

Discussion

The most striking finding in this experiment was the variability of platelet adhesiveness from day to day. As a result of this, changes in platelet adhesiveness attributable to taking the drug would have to be very large in order to be significant. The variability from day to day was also noticeable in the studies into the effects of total starvation and of sucrose. This variability will be discussed in detail later in this thesis. The mean difference in adhesiveness between the 2 periods was 4.1% and in the light

| | Before Treatment | During Treatment. |
|---|------------------|-------------------|
| platelet count x 10 ³ /cmm. | 167.4 ± 40 | 167.9 ± 42 |
| platelet adhesiveness % | 41.2 ± 9.1 | 37.1 ± 5.8 |
| plasma NEFA MEq/l. | 433 ± 90 | 457 ± 104 |
| blood sugar mg/100ml. | 73 ± 4 | 70 ± 4 |
| serum cholesterol mg/100ml | 274 ± 40 | 249 ± 29* |
| serum triglyceride mg/100ml | 94 ± 33 | 93 ± 25 |

TABLE XXIV Effects of Calmic 131A in a dose of 3 capsules/day. The results are the means and standard deviations for 6 subjects.

* - $p < 0.05$

| subject | before treatment | | | | during treatment | | | |
|---------|------------------|------|------|------|------------------|------|------|------|
| | 1 | 2 | 3 | Mean | 1 | 2 | 3 | Mean |
| 1 | 30.7 | 61.4 | 65.3 | 52.5 | 37.3 | 25.3 | 32.3 | 31.6 |
| 2 | 41.1 | 45.5 | 49.0 | 45.2 | 37.9 | 37.1 | 20.5 | 31.8 |
| 3 | 42.3 | 45.4 | 53.0 | 46.9 | 36.4 | 53.0 | 46.1 | 45.2 |
| 4 | 40.8 | 29.4 | 14.7 | 28.3 | 24.1 | 36.7 | 38.0 | 32.9 |
| 5 | 35.5 | 31.4 | 31.0 | 32.6 | 41.7 | 43.7 | 41.3 | 42.2 |
| 6 | 51.8 | 47.3 | 25.4 | 41.5 | 46.4 | 39.5 | 30.4 | 38.8 |
| MEAN | 40.4 | 43.4 | 39.4 | 41.2 | 37.3 | 39.2 | 34.8 | 37.1 |

TABLE XXV Platelet adhesiveness (%) results before and during Calmic 131A administration.

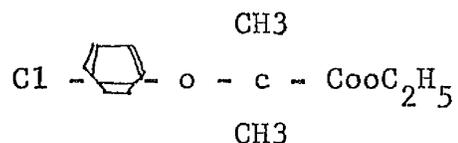
of the day to day variation, is clearly not significant. Millar and Wilson (1968) also failed to find any significant change in platelet adhesiveness due to Calmic 131A. They did however find small insignificant changes in platelet adhesiveness on Calmic 131A in a dose of 6 capsules/day which was associated with a fall in serum cholesterol. There was a significant fall in serum cholesterol in the present experiment but there was no correlation between the changes in serum cholesterol and the changes in platelet adhesiveness. The fall in serum cholesterol is likely to have been due to the administration of unsaturated fatty acids. Seasonal variation is unlikely to have been a factor in such a short term experiment but it should be noted that the experiment was performed in May and June 1966 and that serum cholesterol was tending to fall at this time of the year (Fyfe, Dunnigan, Hamilton & Rae 1968).

Since the withdrawal of Owren's work on linolenic acid, there has not been any support in the literature for the suggestion that platelet adhesiveness might be lowered by linolenic acid. At a clinical level, Christie, Conway & Pearson (1968) have suggested that Calmic 131A improves the performance of subjects with intermittent claudication. Their results were clouded to some extent by a placebo effect but their conclusions appear from their data to be valid. It is difficult to understand what mechanism might underlie the improvement in performance but further investigation to confirm their work would appear to be indicated.

Chapter 17

Effect of clofibrate

Clofibrate is the ester of a short chain fatty acid. Its structural formula is:-



This substance alone or in combination with androsterone has been shown to be capable of lowering serum cholesterol and triglyceride levels and also platelet adhesiveness (Carson, McDonald, Pickard, Pilkington, Davies & Love (1963 & 1966) and Symonds, de Toszeghi & Cook (1964). More recent investigations have failed to demonstrate lowering of platelet adhesiveness however (O'Brien & Heywood 1966; Millac, Caspary & Prineas 1967). The question whether or not clofibrate affects platelets is therefore not established. If clofibrate does lower platelet adhesiveness, it could do so either by direct action on platelets or as a secondary effect via changes in serum constituents such as lipids (Gilbert & Mustard 1963).

The effect of clofibrate in a dose of 1G/day was therefore studied in 6 patients. The average age of the group was 50.5 years (range 40 - 64) and there were 4 males and 2 females. All had ischaemic heart disease. Following admission to hospital, the patients were maintained on the ordinary ward diet. On at least three occasions during the basal period blood samples were taken after an overnight fast with the patient still in bed. Clofibrate (Atromid-S) was then started in a dose of 1G/day and the progress of the subjects followed by taking blood samples under similar conditions to the basal period. The timing of the samples taken while on treatment was variable but can be assessed from Table XXVI which shows all the platelet adhesiveness results for the 6 subjects. Table XXVII shows the mean results during the basal period together with the changes in platelet count, platelet adhesiveness, plasma NEFA, blood sugar, serum cholesterol and serum triglyceride from the mean basal level. The results are shown as differences

| Weeks | Basal | | clofibrate 1G/day | | | |
|-------|--------------|----------------------|----------------------|----------------------|----------------------|------|
| | - 2 | - 1 | 1 | 2 | 3 | 4 |
| S.W. | 43.2 | 47.5 47.4 | - | 45.6 | - | 31.9 |
| A | - | 31.6 29.2 33.6 | 44.7 40.8 | 48.2 33.5 43.8 | 32.2 38.5 42.7 | 37.9 |
| M.W. | 43.9 39.3 | 41.2 37.2 40.0 | 54.4 55.5 | 45.2 35.7 | 35.8 64.4 | - |
| H | - | 30.1 33.7 28.6 | 36.8 35.6 | 40.4 32.4 | - | - |
| M | - | 39.3 29.0 22.3 | 35.3 23.9 23.8 | 36.1 | 37.3 27.8 | 32.5 |
| McD | 56.4 56.6 | 48.3 | 40.7 23.0 | 43.1 52.8 | 46.7 32.9 46.4 | - |

TABLE XXVI Platelet adhesiveness results (%) before and during clofibrate therapy for 6 subjects.

| | Mean Basal | Week 1 | Week 2 | Week 3 | Week 4 |
|---|--------------|------------|--------------|--------------|------------|
| platelet count x10 ³ /cmm | 183.0 ± 18.3 | +1.5 ± 9.0 | +10.1 ± 12.1 | +23.1 ± 12.7 | +9.5 ± 7.1 |
| platelet adhesiveness % | 38.8 ± 4.0 | +1.4 ± 7.0 | +2.6 ± 2.4 | +1.7 ± 4.7 | -1.8 ± 6.4 |
| plasma NEFA MEq/l. | 390 ± 44 | +132 ± 56 | +179 ± 46 * | +43.3 ± 52 | +104 ± 89 |
| blood sugar mg/100ml | 80 ± 6 | -4.5 ± 3.8 | -11.5 ± 4.9 | -10 ± 2.7 | -20 ± 10 |
| serum cholesterol mg/100ml | 355 ± 36 | -30 ± 17 | -40 ± 22 | -27 ± 27 | -32 ± 40 |
| serum triglyceride mg/100ml | 135 ± 12 | -19 ± 11 | -26 ± 7 * | -6 ± 13 | -31 ± 30 |

TABLE XXVII Mean changes (± S.E.M.) from the mean basal level during treatment with clofibrate in 6 subjects.

Difference significant at $p < 0.05$ - *

on this occasion because of the variability in the timing of the venepuncture.

Results and Discussion

The platelet count rose during treatment with clofibrate but this was not significant statistically. A higher platelet count during clofibrate therapy has been demonstrated previously by Gilbert & Mustard (1963), O'Brien & Heywood (1966) and Millac, Caspary & Prineas (1967). Since there does not appear to be any change in plasma volume or total body water during treatment with clofibrate (MacMillan, Oliver, Simpson & Tothill 1965) the increase in platelet count is likely to be a real one. This rise has been attributed to reduced adhesiveness associated with increased platelet survival and reduced turnover (Gilbert & Mustard 1963). In the present instance and also in the cases of O'Brien & Heywood (1966) and Millac, Caspary & Prineas (1967) however, there was no decrease in platelet adhesiveness. The explanation of the rise in platelet count therefore remains obscure.

It is difficult to compare the results obtained by different workers into the effects of clofibrate on platelet adhesiveness because of the many variables. All the references quoted so far have used the Payling Wright method except Gilbert & Mustard (1963). Some workers have used clofibrate alone (Atromid S) others clofibrate with androsterone (Atromid); the states of the patients with regard to fasting and preceding exercise is difficult to assess and is probably varied; the doses of the drug given and the timing of the investigation in relation to the commencement of therapy are also variables. The dose used in the present investigation was low and the measurements of adhesiveness were carried out within a month of commencing therapy. Nevertheless, lipid changes resulting from the drug therapy were apparent and yet there was no alteration in platelet adhesiveness. There is no apparent explanation to account for the fact that some workers have found lowering of platelet adhesiveness while others have not. Lowering of platelet adhesiveness has been found with "Atromid" (clofibrate with androsterone) (Carson et al. 1963; Symonds et al. 1964, Carson et al. 1966) and with "Atromid-S" (clofibrate alone) (Symonds et al. 1964, Carson et al. 1966).

No change in platelet adhesiveness has been found with "Atromid" by Millac et al. (1967) and with Atromid-S by O'Brien and Heywood (1966). The androsterone cannot therefore account for the discrepancy. The results of the present study agree most closely with O'Brien & Heywood (1966) who found an increase in platelet count with no change in platelet adhesiveness. These workers also commented on the variability of platelet adhesiveness for any one subject from day to day, a factor which can be seen in this study also.

Significant depression of the serum triglyceride occurred within 2 weeks of commencing clofibrate. The fall was seen in all subjects except one. Serum cholesterol levels also fell in 5 out of 6 instances but in this case the fall failed to be statistically significant (the subject whose cholesterol did not alter was not the same as that whose triglyceride did not fall). That 1G of clofibrate can produce lipid changes within 2 weeks was established by the investigation involving 11 subjects carried out by Mitchell and Murchison (unpublished) in this department. This rapid onset had previously been observed by Carlson, Hogstedt, & Oro (1963) and Denborough, Lovell & Trevaks (1963) using a combination of clofibrate and androsterone ("Atromid").

The significant finding of elevation of plasma NEFA levels during clofibrate therapy is at variance with the literature on the subject. Several workers (MacMillan, Oliver, Simpson & Tothill 1965 and Rifkind 1966) have shown that when the plasma clofibrate level is taken into account, there is significant depression of plasma NEFA levels. The present study was carried out at a much earlier stage during clofibrate therapy and it would appear likely that the rise in plasma NEFA level is due to the addition of clofibrate (which is extracted along with plasma NEFA by the Dole method) prior to the production of its lowering effect on plasma NEFA levels.

Summary

Clofibrate in a dose which was sufficient to lower serum cholesterol and triglyceride levels and also to raise plasma NEFA levels, did not alter

platelet adhesiveness. Platelet adhesiveness does not therefore appear to be affected by the addition of fatty acid-like materials such as clofibrate and γ -linolenic acid nor by changes in serum cholesterol, serum triglyceride or plasma NEFA produced by these substances.

SECTION IV

REVIEW OF THESIS WITH CONCLUSIONS

Review and Conclusions of Thesis

The aim of this thesis was to explore the possibility of a link between the lipid and thrombogenic theories of atheroma production. The most likely site of this link appeared to be the blood platelet because of the large volume of evidence associating platelets and fatty acids in vitro. The bulk of this thesis has been an investigation to determine if this link is present in vivo. Platelet adhesiveness was chosen as the measure of platelet function because it had been shown by several workers to be elevated in ischaemic heart disease and because the published evidence linking fatty acids and platelets had incriminated the ADP mechanism, red cell integrity and the early surface active stages of coagulation. Platelet adhesiveness would be expected to be an indicator of acceleration of any of these components and therefore a good measure of the existence of such a link.

The measurement of platelet adhesiveness by the Payling Wright method is technically very simple and its accuracy depends on platelet counting. The method described for platelet counting using ammonium oxalate to lyse the red cells and also phase contrast microscopy, proved to be satisfactory. The accuracy of the method was shown to be comparable to that of other workers. The platelet count although steady under basal conditions is influenced by very simple things such as exercise or smoking a cigarette. Drugs too have been shown to alter it. Noradrenaline causes an increase while stimulation of beta-adrenergic receptors with isoprenaline surprisingly causes quite a marked decrease. Over the longer term, clofibrate appears to produce an increase in the platelet count. The platelet count for any individual varies randomly from day to day. This variation corresponds well to that described in the literature. For example, in the investigations into the effect of clofibrate and Calmic 131A (12 subjects; average of 8 counts each) the average difference between the highest and lowest platelet counts for each subject was 43,000/ cu.mm. This is comparable to 30,300/ cu.mm. (4 subjects; 4 counts each) obtained from the data of Sloan (1951). Over a longer period such as that involved in the sucrose study, the figures were again comparable. The average difference between the highest

and lowest counts for each subject in this study was 96,400/mm (approx. 20 counts) compared to 106,400/mm (32 counts) from the data of Brecher, Schneiderman & Cronkite (1953). Thus the longer an investigation continues, the greater will be the variation in platelet count for any individual. This is due to the fact that the number of counts and therefore the chance of errors arising due to inaccuracies in counting are increasing. However, in addition to the errors in platelet counting, other variables will be contributing, such as the state of hydration (as shown by the fasting experiment), exercise or smoking.

Platelet adhesiveness has been shown to be stable under basal conditions over short periods of time but like the platelet count it is affected by many commonly occurring stimuli. Minor stress in the form of embarrassment (puffing an unlit cigarette) appears to produce an effect. Stress of greater intensity such as a surgical operation or the infusion of noradrenaline also produces an increase in adhesiveness. Exercise and the ingestion of fat or glucose are also followed by increased platelet adhesiveness. The conditions under which platelet adhesiveness is measured should therefore be strictly controlled. Subjects should be at rest for at least half an hour and should be fasting. A point which seems to have been overlooked by other workers using the Payling Wright method is that the interval between venepuncture and the commencement of the measurement should be controlled. In the present series of experiments this variable accounts for the differences in mean basal platelet adhesiveness between some experiments. The measurement was carried out 5 minutes after venepuncture in the studies into the effect of smoking, noradrenaline and isoprenaline, glucose, sucrose, Calmic 131A and clofibrate. The investigations into the effects of surgery, starvation and fat on the other hand were carried out 20 minutes after venepuncture while that on exercise was carried out 3 minutes after venepuncture. The reason for these different intervals was that in the 20 minute cases, delays were expected with some samples due to patients being in other wards of the hospital, while in the exercise experiment 3 minutes was chosen to fit into the design of the experiment in order that all the different manoeuvres might be performed.

Adhesiveness levels are therefore only comparable in the experiments in

which the interval between venepuncture and measurement is the same. Due to a lack of numbers, platelet adhesiveness in subjects with ischaemic heart disease was not statistically higher than the others. If however, the subjects with ischaemic heart disease from the other experiments in which the time between venepuncture and measurement was 5 minutes are added in to the results already quoted, the difference becomes statistically significant (Table XXVIII. Fig 15.).

Figure 15.

Individual results for platelet adhesiveness for subjects with and without ischaemic heart disease in Table XXVIII.

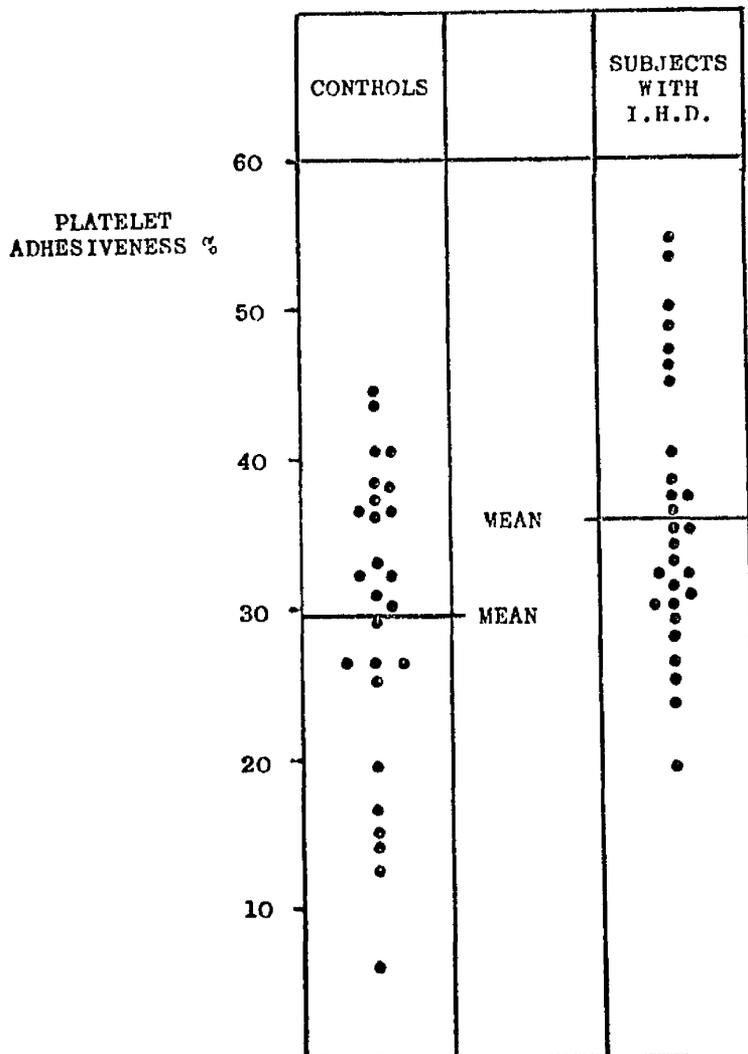


TABLE XXVIII

Mean platelet adhesiveness for all subjects with ischaemic heart disease included in the thesis compared to that of the subjects without clinical evidence of ischaemic heart disease from Figure 3.

| | ischaemic heart disease | others |
|-----------------------|-------------------------|--------------------|
| number of subjects | 28 | 26 |
| males/females | 23/5 | 12/14 |
| average age | 56.7 years | 44.2 years |
| platelet adhesiveness | 36.1% | 29.5% ⁺ |

+ $p < 0.02$.

TABLE XXIX

Comparison of platelet adhesiveness (measured 5 minutes after venepuncture) in all subjects with ischaemic heart disease with those without clinical evidence of ischaemic heart disease.

| | ischaemic heart disease | others |
|-----------------------|-------------------------|--------------------|
| number of subjects | 28 | 55 |
| platelet adhesiveness | 36.1% | 31.2% [*] |

* $p < 0.05$.

The two groups it should be emphasised, are not age and sex matched, the ischaemic heart disease group containing 23 males and 5 females with an average age of 44.2 years. As pointed out already however there did not appear to be any difference in adhesiveness between the sexes or any increase with age. The difference in platelet adhesiveness is also significant if the subjects without ischaemic heart disease in the comparable experiments are added to the "control" group. This merely increases the size of this group and makes the "control" group twice as large as the other (Table XXIX).

In the conditions investigated which predispose to ischaemic heart disease, there was no evidence of increased platelet adhesiveness. Obese subjects did not have increased adhesiveness and weight loss did not result in any significant change in adhesiveness. There was no acute change in adhesiveness following cigarette smoking. The effect of chronic cigarette smoking cannot be assessed from the experiments carried out but it is interesting that the mean basal levels in the subjects involved in the study (all smoked at least 10 cigarettes/day) were slightly higher on average than in any other comparable experiment. Adhesiveness in subjects with hypercholesterolaemia or hypertriglyceridaemia was not formally investigated but it can be said that when the serum cholesterol or serum triglyceride was significantly altered there were no corresponding changes in platelet adhesiveness nor was there any relationship between the levels of cholesterol or triglyceride and platelet adhesiveness. Sucrose has been put forward as an aetiological factor in ischaemic heart disease but here again, taking the results over all, there did not appear to be any increase in platelet adhesiveness over a period of 1 month on a sucrose diet.

There are considerable difficulties attached to investigating platelet adhesiveness changes over a prolonged period of time. The variation in platelet adhesiveness is much greater than that obtained for duplicates and during short term experiments even when great care is taken to standardise the conditions under which the blood samples are obtained. This variation means that very large changes in platelet adhesiveness would have to occur in response to specific stimuli in order to be statistically significant.

Most workers tend to hide individual results by presenting averages and so it is difficult to assess the variation found by others. O'Brien & Heywood (1966) stated that the variation was considerable but did not quote figures. Symons, de Toszeghi & Cook (1964) in their investigation in the effect of clofibrate show all their results. Spontaneous variations of up to 23% adhesiveness in one subject are apparent during the control period. Besterman et al. (1967) shows graphs of results in 4 subjects over a period of months and the variation here again is 25% adhesiveness in one of the subjects. This represents around a quarter of the total adhesiveness scale and when one realises that in practice most platelet adhesiveness results occur within a limited range of about 40% of the total scale the practicability of undertaking any long term study involving platelet adhesiveness must be seriously questioned.

The present series of investigations have been unsuccessful in establishing a definite link between platelets and fatty acids in vivo. A great many stimuli have been found to raise both platelet adhesiveness and plasma NEFA. These include surgery, noradrenaline infusion and also sham smoking. There was no correlation between the fatty acid and platelet changes except in the sham smoking/smoking experiments. Platelet adhesiveness has also been shown to rise following stimuli which lower plasma NEFA (glucose and exercise) and which do not alter plasma NEFA significantly (isoprenaline). In addition platelet adhesiveness was unchanged following fasting when the plasma NEFA levels were substantially elevated. It is clear that a rise in platelet adhesiveness can be associated with plasma NEFA changes going in either direction or indeed remaining relatively unchanged while platelet adhesiveness can be unchanged in spite of a rise in plasma NEFA. Thus there is no apparent association between changes in platelet adhesiveness and plasma NEFA or if there is, other more powerful influences have hidden the association. This is in agreement with Bridges, Dalby, Hadden, Johnston & Weaver (1967) who studied the effects of growth hormone and nicotinic acid on platelet adhesiveness and NEFA. The positive correlation demonstrated in the smoking and sham smoking experiments is unlikely therefore to be valid in view of the failure to demonstrate it in other situations.

One might argue that the effects of fatty acids on platelets might not become apparent until all the tight binding sites for fatty acids on albumin had been saturated (i.e. at about 1212 μ Eq/l.) the reason being that only at above this level would fatty acids be free to adhere to cell walls. This level was exceeded in the fasting experiment in 8 out of 16 subjects and yet no effect on the platelet count or platelet adhesiveness was noticed. It must be conceded that the levels of fatty acids were being overestimated due to the extraction of ketone bodies along with the fatty acids in this experiment. However in the 8 instances, in which the fatty acids were doubly extracted, the level of 1212 μ Eq/l. was exceeded twice without any effect on platelets or clinical evidence of thrombosis.

Since fatty acid transport is so rapid it would not be unreasonable to expect the second class binding sites on albumin and on cell walls to be important in fatty acid transport. After all, how could fatty acids be rapidly removed from tight binding sites on albumin and into cells without involving the second class binding sites on the cell wall? The plasma NEFA level in fact does not indicate the rate of turnover of fatty acids, merely the balance between formation and removal. Could it be then that platelet adhesiveness changes might be related to the rate of turnover of fatty acids through binding sites on cell walls? There is evidence against this. Platelet adhesiveness was increased when plasma NEFA levels were reduced due to reduction in turnover following oral glucose administration and also due to increase in turnover following exercise.

In view of the in vitro evidence suggesting that unsaturated fatty acids may protect platelets from the effects of saturated acids, it could be argued that the proportion of unsaturated to saturated fatty acids is the factor determining platelet adhesiveness. Here again, the evidence from the sham smoking experiment makes this unlikely since an increase in the proportion of unsaturated fatty acids was accompanied by an increase in platelet adhesiveness. Also the administration of unsaturated fatty acids in Calmic 131A in a dose sufficient to lower serum cholesterol did not significantly affect platelet adhesiveness. Clofibrate which has structural similarity to a fatt

acid did not affect platelet adhesiveness when given in a dose sufficient to alter serum lipid levels. Thus, investigations launched from different fronts, have failed to reveal any relationship between plasma NEFA and platelet adhesiveness in vivo. The link between fatty acids and platelets postulated in previous in vitro studies cannot be substantiated by the present in vivo experiments. All the in vitro fatty acid preparations, however ingeniously they have been compounded, are completely unphysiological and therefore do not appear to have any "in vivo" significance.

It has been suggested that blood glucose may play some part in determining platelet adhesiveness, thus explaining why increased adhesiveness has been found in diabetics (Bridges, Dalby, Millar & Weaver, 1965). The increase in adhesiveness following oral glucose has been confirmed but there was no correlation between the changes. A rise in blood glucose following noradrenaline, isoprenaline and oral fat was accompanied by a rise in adhesiveness also, but again there was no correlation between the two changes. Exercise on the other hand caused an increase in platelet adhesiveness even though the blood sugar did not alter while the subjects with the most marked rises in blood sugar following cigarette smoking showed decreases in platelet adhesiveness. A fourth combination, namely a significant fall in blood sugar with no change in platelet adhesiveness, was found in the fasting experiment and this makes any correlation between blood sugar and platelet adhesiveness changes very unlikely. One might suggest that both plasma NEFA and blood sugar might be affecting platelet adhesiveness but this does not bear critical examination since a rise in both have been associated with both an increase (noradrenaline infusion) and a decrease (Smoking experiment) in platelet adhesiveness.

Therefore, no evidence has been found to link platelet adhesiveness with levels or changes in plasma NEFA, blood glucose, serum cholesterol or serum triglyceride in vivo. What does control platelet adhesiveness? Circulatory changes appear unlikely because a rise in adhesiveness was accompanied by a bradycardia in the noradrenaline experiment and by a tachycardia in the isoprenaline and exercise experiments while the blood pressure rose in the noradrenaline experiment but was unaffected in the isoprenaline experiment.

Catecholamines, in view of the fact that adrenaline and noradrenaline can cause platelet aggregation in vitro, might be held responsible in these experiments for the increase in adhesiveness. Catecholamine release probably occurred in many of the other situations in which a rise in adhesiveness occurred, for example in the sham smoking and exercise studies. However there has never been any suggestion of catecholamine release following the ingestion of fat or glucose. Catecholamines might be expected to be released as a result of the stress of venepuncture. The pattern of change in platelet adhesiveness in the experiments does not support this idea since one would expect changes to have occurred during the basal periods if this was a significant factor and this did not occur.

As platelet adhesiveness is such a labile property it would appear likely that no one factor controls it. Like ischaemic heart disease, it probably has a multifactorial control mechanism although as demonstrated in this thesis the factors are likely to be different from those causing ischaemic heart disease.

No relationship has been found between serum lipids and platelet adhesiveness. It has therefore not proved possible to link the thrombogenic and lipid theories of atheroma production via platelet adhesiveness. The role of the increased platelet adhesiveness found in ischaemic heart disease remains in doubt but it remains a cornerstone of the evidence supporting the thrombogenic theory.

SECTION V

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