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Drug and dietary modification of platelet function

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Determination of MDA released from platelet aggregation: 200 ul PRP was incubated with 20 ul AA at 37 degrees C with continuous stirring for 5 minutes.Test samples were treated in the same way as for the standard curve.

Concentrations of MDA produced in the test sample were interpolated from the STD curve. Results were expressed as nmol of MDA per 10^9 platelets using the following formula:

$$\frac{\text{MDA} \times 10^6}{\text{Platelet count} \times 2}$$

3.5 Thromboxane B_2 Assay

3.5.1 Sample collection and storage

All samples were processed immediately after aggregation had occurred and assayed as soon as possible. Samples were centrifuged at 2000 g for 30 minutes at 4 degrees. The plasma fraction was then isolated and stored at - 20 degrees C until needed.

3.5.2. Assay procedure

Thromboxane B_2 in PPP was measured by radio-immunoassay (Ferraris et al., 1977), using tritiated thromboxane B_2, thromboxane B_2 antibody and thromboxane B2 standard concentrate as supplied by New England Nuclear Boston, Mass, (cat. No NEK-007).

Details of the method used were in accordance with the instruction manual supplied by the company. Additional plasma blanks using each patient's own plasma in order to compensate for colour quenching (self absorption) by individual plasmas were used.

During the assay it was found to be convenient to add the buffer and standards or unknowns first to all the tubes before the addition of antibody and tracer. The standards were prepared as serial geometric dilutions in buffer containing 0,01% gelatin. The gelatin acts as a carrier in the subsequent precipitation of the antigen - antibody complex, and it coats polar groups in the glassware and thus prevents the adsorption of immunoglobulins to the vessel walls.

After addition of the antibody, the tubes were vortexed and left for 60 minutes prior to the addition of the labelled ligand or tracer. This is known as the 'delayed addition of tracer' technique (Granstrom & Kindahl, 1978), which increases the sensitivity of the assay. The tracer or ligand was then added. After a careful final mixing of the tube contents, the whole set of tubes were incubated at 4 degrees C for 16 hours. After the incubation period, separation of the antibody bound and free fractions was accomplished by the addition of dextran coated charcoal. This technique is

based on the removal by the charcoal of the free molecules from the incubation; however, if the time allowed for this adsorption becomes too long, the antigen-antibody complex dissociates, more free antigen is adsorbed, and the final radioactivity measurement of the bound fraction becomes too low. This becomes a greater problem as the number of tubes in the assay increases, since they obviously cannot all be handled simultaneously. In order to avoid irreproducible stripping of counts upon exposure to charcoal, the charcoal suspension was added to only as many tubes as could be centrifuged within thirty minutes following the addition of charcoal to the first tube.
After the addition of charcoal the tubes were centrifuged at 1800 g for 10 minutes. The supernatant was then decanted into liquid scintillation (LS) vials and 10 ml LS cocktail added.

The protocol from the kit suggested the use of atomlight or Pica fluor scintillating cocktail. However, these cocktails were not available and problems with chemical luminescence (increased transfer of energy from the beta-particle through the solvent/scintillator system to the photomultiplier) were experienced with Instagel. Dimilume 30 (Packard, cat. no 6013067) which contains a chemiluminescence inhibitor was therefore used. All samples were counted on a Packard Tri-Carb 2660 scintillation counter.

In the present study, cross reactivity to AA was checked in the following way - antibody was incubated with a constant amount of radiolabelled antigen, and attempts to inhibit the achieved binding was made by adding arachidonic acid in increasing amounts. Arachidonic acid was found to have a cross reactivity of `0.1%.

3.5.3 Calculation of unknowns

The following method was used for the calculation of the standard curve and the unknowns:

1.All raw counts were corrected to counts per minute (cpm).

2.The counts for each set of duplicates were averaged.

3.The net counts for all standards and samples were calculated by subtracting from each the average of the appropriate blank (see table 3.2)

4.The percent bound (%B) for each standard and sample were determined by dividing the average net cpm by the average of the total count tubes. It is very important to note that, by convention, blank counts are not considered to enter into the antibody-antigen reaction. Since the presence of plasma in the reaction tube causes a significant change in the blank it is necessary to calculate a net total count number to properly account for the different sample matrices. The standard curve must be calculated using buffer net total counts and the plasma samples must be calculated using

plasma net total counts.

5.The normalized percent bound (% B/Bo) for each standard calculated as follows:

$$\%B/Bo = \frac{\%\ B \text{ of Standard}}{\%\ B \text{ of '0' Standard}} \times 100$$

6.The normalized percent bound (% B/Bo) for the human plasma controls were calculated as follows:

$$\%\ B/Bo = \frac{\%\ B \text{ of Control 1.0 ug/ml added}}{\%\ B \text{ of Control, 0 ug/ml added}} \times 100$$

7.The normalized percent bound (% B/Bo) for the samples were calculated as follows:

$$\%B/Bo = \frac{\%\ B \text{ of Plasma (Patient) sample}}{\%\ B \text{ of Control, 0 ug/ml added}} \times 100$$

8.Using semi-logarithmic paper, % B/Bo for each standard versus the corresponding picogram (pg) thromboxane B_2 added, was plotted. The pg thromboxane B_2 in each sample was determined by inter-polation from the standard curve. Since all samples (plasma or buffer) were normalized, plasma samples were read from the buffer standard curve. Since identical volumes were used for standards and samples and the standard curve was expressed as pg Thromboxane B_2 added, samples were read directly as pg thromboxane B_2

The working range of the standard curve was found to be between 10-100 pg. This constituted the

straight line of the curve. All values below the lower limit of detection (10 pg) was regarded as 0 because of the inaccuracy of the curve.

Table 3.2 Table showing sample and appropriate blank

Sample	Appropriate Blank
Standard Solutions	Buffer Blank
Standard Plasmas	Human plasma blanks supplied in the kit
Patients Plasmas	Patients Blanks

3.6 Beta-thromboglobulin (B-TG)

3.6.1. Preparation and storage of plasma samples

Since B-TG is released by blood platelets when they undergo their release reaction, which is an essential stage in the blood clotting process, a serum sample is not acceptable for this test. Platelet release may also occur during the collection and processing of citrated plasma. For this assay, PPP, processed so as to minimize platelet release during handling, must be used.

The sampling protocol for the B-TG RIA has been developed to achieve this purpose as simply and conveniently as possible. The protocol is a modification of that recommended by Ludlam and Cash (1976). The system employs a mixture of anti-coagulant (EDTA-10mmol/L) and antiplatelet reagents (Theophylline-5.4 mg/ml and PGE_1-1.1 mg/ml) which, when in combination with a blood sample maintained at reduced temperature, serve to minimize the amount of B-TG release by platelets *in vitro* during processing. In this way, the measured B-TG value reflects the *in vivo* circulating level at the time of sampling.

Sample preparation was the critical stage in this assay and the following protocol was strictly adhered to:

1. A 2.5 ml venous blood sample was withdrawn from the patient using a 19 gauge needle attached to a syringe. A good clean venepuncture with minimal venous occlusion was performed. The period of venous occlusion during collection was always less than 2 minutes.
2. Immediately after collection, the blood was transferred to one of the blood sampling tubes (containing EDTA-10 mmol/L, theophylline-5.4 mg/ml and PGE_1-1.1 mg/ml). The tube was pre-cooled in a crushed ice-water bath.
3. The tube was stoppered and the contents mixed by two or three gentle inversions. The tube was then

immediately cooled by placing it in a crushed ice-water bath.

4. Sufficient time was allowed for the sample to cool (about 15 minutes). The ice-water bath was maintained at 0 degrees C throughout this time.

5. The sample was centrifuged at 2000 g at 2-4 degrees C for 30 minutes in an MSE refrigerated centrifuge. Centrifugation was carried out within half an hour of blood collection.

6. After centrifugation, the top 0.5 ml of the supernatant was carefully removed and transferred to a polypropylene tube.

7. The sample to be assayed was stored at -20 degrees C until needed. Samples were assayed after thawing and were never re-frozen for further assays.

3.6.2. Assay procedure

B-TG was measured by RIA based on the method originally described by Ludlam et al., (1975), using B-TG ^{125}I tracer, B-TG antibody and BTG standard concentrates as supplied by Amersham International. Details of the method were in accordance with the instruction manual supplied by the company.

Before reconstitution of the standards, tracer and antibody, care was taken to tap each vial on the bench top to bring the contents to the bottom of the vial.

When the stopper was removed, precautions were taken not to dislodge any small particles of freeze dried material adhering to the stopper. After the addition of distilled water, the stoppers were replaced and the contents of the vial were mixed by gentle swirling and inversion until an homogenous solution was obtained. All standards used were supplied in known quantities eg. 10, 20, 50, 100 and 225 ng/ml in the kit and thus preparation of the standards was not necessary.

B-TG RIA utilizes a saturated ammonium sulphate solution to separate the bound labelled B-TG from the free B-TG. After incubation of the antigen and antiserum, a standard amount of ammonium sulphate solution was added and vigorously agitated with the mixture. The precipitate was then separated by centrifugation, 1500 g for 15 minutes.

The supernatant was discarded and the precipitate counted to determine the amount of ^{125}I B-TG bound to antiserum. This amount will vary inversely with the quantity of non-labelled antigen present in the original known (standard) or unknown (sample) mixture. When the supernatant was discarded, the tubes were gently inverted in one continuous movement so that the precipitate remained undisturbed at the bottom. Tapping and shaking of the tubes were avoided during

decantation. Once the supernatant was poured off, the tubes were stood upright on paper tissue until draining was complete. After draining, the rims of the inverted tubes were gently tapped on the paper tissue to remove any liquids remaining on the rims. All samples were counted in a Packard Gamma Scintillation spectro-meter model 578 for 10 minutes.

When counting the radioactivity, the use of glass tubes for the secondary containment of the counting tubes was avoided, since the attenuation of the low energy ^{125}I-gamma-radiation by glass is relatively high. Not only is the count rate markedly reduced, but serious errors may arise, due to variations in the wall thickness of the glass container. Where secondary containment was required, moulded plastic vials were used.

3.6.3 Calculations of unknowns

A curve of ^{125}I counts for the five standards against the B-TG concentration printed on the standard vials, was plotted on linear graph paper. A smooth curve was drawn through the duplicate points and grossly aberrant points were rejected. Using the means of the duplicate counts for the unknowns, their B-TG concentrations were read off from the standard curve.

3.7 Circulating platelet aggregates

Circulating platelet aggregates were measured according to the method of Wu and Hoak (1974). Venous blood was drawn directly into two separate polypropylene syringes, one containing 2ml buffered EDTA/formalin solution and the other buffered EDTA solution only. The buffered EDTA/formalin solution was prepared by adding 3ml of 0.077m EDTA, 5ml of 4% formalin, and 2ml of concentrated (10x) phosphate-buffered saline solution (PBS) to 10ml of distilled water. The buffered EDTA solution was prepared by adding 3ml of 0.077m EDTA and 5ml of concentrated (10x) PBS to 12ml of distilled water. Both solutions were isotonic with a pH of 7.4. After blood was drawn into the syringes (in duplicate), they were thoroughly mixed and transferred into polyethylene tubes.

The samples were kept at 25 degrees C for 30 minutes and then centrifuged at 150g for 7 minutes to obtain PRP. Platelet counts on both samples were determined using a thrombocounter C(Coulter Electronics). The mean of the results was expressed as follows: Platelet aggregate ratio = platelet count in EDTA formalin PRP/platelet count in EDTA PRP

3.8 Platelet survival studies

Platelet survival was measured using Indium labelled

platelets by the method of Thakur et.al., (1976) as modified by Heyns et.al., (1980)

3.8.1 Platelet labelling

Autologous platelets were labelled under aseptic conditions. Blood (50ml) was taken by clean venepuncture using a syringe fitted with an 18 gauge needle. The blood was transferred to a sterile 50ml screw capped tube containing 7.5 ml acid-citrate dextrose (ACD) solution as anticoagulant. After mixing by inversion, the tube was centrifuged at 10 g for 15 minutes to generate platelet rich plasma (PRP). The PRP was centrifuged at 1800 g for 10 minutes to obtain platelet poor plasma (PPP) and a platelet button. The PPP was retained aseptically. The platelet button was washed with saline (3x) to remove plasma and then resuspended in 2.5ml saline. An appropiate amount of indium oxine was then added dropwise to the platelet suspension. After a 30 minute incubation period at 37 degrees C, 10 ml of PPP was added to the platelet poor suspension, mixed by inversion and centrifuged at 1800g for 10 minutes. The supernatant was discarded and the platelets were resuspended in 10ml of PPP. Approximately 2ml of the platelet suspension was retained to use as a reference standard and the remaining suspension was transferred to a syringe. The syringe containing the labelled cells was weighed before

and after intravenous administration of the platelets and the weight of the injected dose determined by difference.

3.8.2 Recovery studies

A one hour postinjection blood sample (anticoagulated with EDTA) was taken to determine the fraction of labelled platelets remaining in the circulation (recovery) and calculated using the activity per ml of each sample; the estimated blood volume (i.e. 7.5% of body weight) as shown by Nadler et.al.,(1962) and the formula:

$$\% \text{ Recovery} = \frac{\text{blood volume x activity/ml at 1 hour x 100}}{\text{activity of STD/ml x Xml injected.}}$$

The samples were counted in a NaI crystal well scintillation detector.

3.8.3 Platelet survival studies

Sequential 5ml blood samples (anticoagulated with EDTA) were taken daily for up to 10 days. The samples were counted for radioactivity and the results expressed as a percentage of the 1 hour postinjection sample. Linear regression analyses were carried out, relating percentage of the 1 hour sample remaining in the circulation with time. The extrapolated line of best fit crosses the ordinate at a point which represents

increased loss of platelets between the 1 hour and subsequent samples. The survival can be calculated by extrapolation of the line to zero percent or from the half time (survival = $T^{1/2}$ x 1.44) The slope of the regression line is a measure of the mean loss per day.

3.9 Lipid Extraction Procedure

The method used was the classical method described by Folch et al., (1957). The fish tissue and platelets were added to approximately ten volumes chloroform-methanol 2:1 (v/v). The fish tissue was homogenised using a high speed homogeniser (Ultra-Turrax, Janke and Kunkel K.G. Germany). The washed platelet button was sonicated for 30 sec. in an ice bath using an MSE-150 watt ultrasonic disintegrator Mk2. After rinsing the latter, the extract was made up to twenty volumes with chloroform-methanol (2:1), mixed well and centrifuged. The supernatant was removed to a clean centrifuge tube and mixed thoroughly with 0.2 of its volume of 0.73% NaCl. After centrifuging at 1500g for 10 mins, the lower lipid containing layer was removed and filtered through washed filter paper. The upper phase was re-extracted by adding 0.66 volumes of pure lower phase (chloroform-methanol-water, 86:14:1, v/v), mixing and recentrifuging. The lower phase was removed and filtered. The pooled lower phase was then washed three times by adding 1.5 volumes of pure upper phase

containing 0.29% NaCl (chloroform-methanol-0.58% NaCl, 6:96:94, v/v). After mixing well and centrifuging the upper phase was pipetted off and discarded. Finally, the lower phase and remaining upper phase were made into one phase by adding methanol. Extracts were stored in Teflon-lined screw cap tubes at -20 degrees C.
All solvents contained the antioxidant 2,6-di-tert-butyl-4-methylphenol, 0,005%.

3.9.1 Fatty acid analysis

Fatty acid profiles were determined on whole lipid extracts of fish tissue and platelets. The method of Morrison and Smith (1964) for the methylation of fatty acids were used in this study. One millilitre aliquots of the extracts were placed into glass tubes, and 0,7 ml boron trifluoride-methanol added. The tubes were sealed with inert stoppers, and placed in a sand bath at 95 degrees C for 75 minutes to obtain fatty acids derived from triglycerides, phospholipids as well as sphingolipids. After cooling in water, 0.8 ml hexane and 1 ml saturated NaCl solution was added. The tubes were shaken, and centrifuged at 1500g for 5 mins until the hexane layer was clear. The hexane layer was removed, evaporated down under N_2 and the residue dissolved in a suitable volume of hexane depending on the fatty acid concentration prior to GC analysis.
The procedure of Morrison and Smith (1964) was chosen

for the methylation because it lacked a separate hydrolysis step and was thus quicker to perform. Since boron trifluoride-methanol behaves like a strong acid, hydrolysis and methanolysis were readily achieved by one reagent

3.9.2 Gas liquid chromatography

Fatty acid methyl esters were separated on a 10% SP 222 PS column (6' x 0,25" I.D., Supelco Inc., USA) in a Packard Becker 417 chromatograph fitted with a flame ionisation detector. A temperature gradient of 170-175 degrees C and a nitrogen flow rate of 30 ml/min was employed. Fatty acids were identified by comparing retention times with those of known standards, and the areas under the peaks were computed using an integrator (Autolab Minigrator Spectro-Physics, USA).

The dimethylacetals (DMA) obtained from fatty aldehydes were identified after GLC and excluded from the calculations.

3.10 Serum Cholesterol

All lipograms were done on a Gemeni Mini centrifugal analyzer in the Department of Chemical Pathology, SAIMR under the supervision of Professor D. Mendelsohn.

3.11 Statistical Analyses

Since there was strong doubt whether any of the variables were normally distributed, nonparametric tests were employed.

For the paired observations Wilcoxon's test was used when the data was considered as coming from a continuous distribution and the sign-test was used in cases where the data had too many tied observations. When the different groups were compared, the U-test of Mann-Whitney was used for continuous data. When the data was not continuous it was categorised and a X2-test was performed on the 2 x 2 tables. In the latter case when the sample size was too small Fisher's exact test was used. All these test statistics are fully described by Siegel (1956).

CHAPTER 4

DOSAGE FREQUENCY FOR SUPPRESSION OF PLATELET FUNCTION BY LOW DOSE ASPIRIN THERAPY

4.1. Introduction

The therapeutic use of aspirin with regard to its platelet antiaggregatory activity has been based on the hypothesis that increased platelet-vessel and platelet-platelet interactions are causative factors in the development of arterial and venous thrombi, as well as thromboembolic phenomena (Marcus, 1983; Preston and Greaves, 1985).

Theoretically, inhibition of the ability of the platelets to aggregate at a site of injury should diminish the size of the thrombus and reduce the number of emboli that can be formed from it. Since aspirin inhibits platelet cyclo-oxygenase and thereby prevents the formation of the prostaglandin endoperoxides (PGG_2 and PGH_2) and thromboxane A_2 (Roth and Majerus, 1975; Smith et al., 1977; Ali and McDonald, 1978), there is reason to believe that aspirin might modify the thrombotic process. In animal experiments it has been shown that in some circumstances aspirin diminishes the amount of thrombus formation at a site of vessel injury. (Didisheim and Fuster, 1978).

4.1.1. Clinical Evaluation of Aspirin As An Antithrombotic Agent

4.1.1.1. Aspirin and Coronary Heart Disease

Platelets are involved in the genesis of ischaemic heart disease by incorporation into occlusive coronary artery thrombosis, by embolization of aggregates into the microcirculation of the heart and by their contribution to the production of atherosclerosis (Mustard and Packham, 1975; Mustard et al., 1981; Preston and Greaves 1985; Sherry 1985). There is also increasing evidence that coronary artery spasm may be caused by the release of vasoconstrictors from platelets such as thromboxane A_2 (Moncada and Vane, 1979) and this may result in myocardial infarction. These considerations have led to an active and continuing interest in evaluating the effect of drugs which have the ability not only to suppress platelet aggregation but to be active in inhibiting thrombus formation in experimental animals (Sherry, 1985). Since multiple and complex pathways are involved in the initiation, regulation and control of platelet aggregation, it is not surprising that many drugs have been shown to affect platelet aggregation under selective conditions; furthermore, newer ones with perhaps greater potential are being discovered as we learn more about basic mechanisms of platelet function,

the regulation of vascular phenomena, and the interaction between platelets and the vessel wall. (Sherry, 1985). The next section deals with a review of the effects in major clinical trials of the first generation anti-aggregating agent, aspirin, which is arguably the most important (Preston and Greaves, 1985).

4.1.1.1.(a) Clinical Studies

Two series of case-controlled retrospective studies seeking relation between aspirin ingestion and coronary artery disease have yielded conflicting results. The report by the Boston Collaborative Drug Surveillance group (1974) concluded that a negative association existed between regular aspirin ingestion and death from myocardial infarction. However, a more extensive study, that included over one million subjects found no association between regular aspirin ingestion and a reduced incidence of myocardial infarction (Hammond and Garfinkel, 1975). Six prospective studies have tested the hypothesis that aspirin protects patients with myocardial infarction from recurrent infarction or death. The first of these was reported by Elwood et al., (1974). This study was a multi-centre, randomized, double blind trial of aspirin in doses of 300 mg per day. Male patients were entered following myocardial infarction (mean entry date 10 months post

infarct) and followed up for 30 months. Overall, there was a non-significant trend for decrease in the mortality of the aspirin-treated group. However, in those patients who entered the study within 6 months of infarction, the mortality rate was 7.8% in the aspirin group, compared with 13.2% in the placebo group. This suggested that the patients who were treated within a short time of the infarction were more responsive to aspirin treatment. Thus, a subgroup who might be more responsive to aspirin treatment was identified.,

In 1976, the Coronary Drug Project Research Group reported their results in 1529 males who had suffered a previous episode of myocardial infarction and were randomized to receive either aspirin (324 mg t.i.d.) or placebo. All patients had an interval of at least 2 years between myocardial infarction and entrance into cases, this interval was more than 5 years. The patients were followed from 10 to 28 months, and the mortality in the aspirin group was 5.8% compared to 8.5% in the placebo group. Once again, there was a trend towards decreased mortality in the aspirin group, but this was not statistically significant.

Breddin et al., (1979) reported the results of a German-Austrian study which showed a strong trend in

favour of aspirin compared with the placebo or an anticoagulant-treated group, but again the results were not statistically significant.

Elwood and Sweetnam (1979) reported the results of a double-blind trial of aspirin in doses of 900 mg per day in patients who were admitted to the study within seven days of infarction. The mortality was 12.3% in the aspirin-treated patients and 14.8% in the placebo-treated group. This difference was not significant. In a male subgroup analysis aspirin significantly reduced the total mortality and secondary occurrence of myocardial infarction in males. However, there was a 25% non compliance and a high withdrawal rate in this trial which may negate the results.
Two multi-centre trials have recently been reported in the United States. The Aspirin Myocardial Infarction Study (AMIS) 1980, was a prospective trial in which patients were randomized into aspirin or placebo treated groups eight weeks to five years following myocardial infarction. There were 2067 patients in the aspirin-treated group and 2257 in the placebo-treated group. Over a three year follow-up
period, there was no significant difference in the overall mortality, cardiac related mortality or recurrence of myocardial infarction. There was a non-significant trend towards a reduction in the

occurrence of stroke and transient cerebral ischaemic attacks in the aspirin treated group. The lack of benefit provided by aspirin does not appear to support the observations initially reported by the Boston Collaborative drug surveillance group (1974). However, the mean interval between the qualifying myocardial infarction and entry for each group was 25 months, so that this study may have omitted the group of patients most likely to respond to antiplatelet agents. A large percentage of patients in this study were also of relatively low risk, since it is well recognized that the mortality rate is highest within the first four months of myocardial infarction (Pell and D'Alonzo 1964; Beard et al., 1960). Thus, the negative findings in the Amis study may have been due in part to the inclusion of a large percentage of low risk and low responsive patients (Kelton and Hirsch, 1981a,b).

The Persantine Aspirin Reinfarction study group (PARIS) (1980), reported the results of 2026 persons who had recovered from myocardial infarction. They had been randomized into three groups: aspirin plus persantine (dipyridamole) (n=810), aspirin alone(n=810)or placebo (n=406). The average length of follow up was 41 months. The result showed a non-significant trend in favour of aspirin for the prevention of total mortality and

sudden death in patients with previous myocardial infarction.

The Enquete de Prevention Secondaire de l'Infarctus du Myocarde (EPSIM) (1982), reported the results of the EPSIM trial. In this study they compared the effects of aspirin (500 mg t.i.d.) and oral anticoagulants in 1303 men and women who had a documented myocardial infarction. They were randomized to anticoagulant (652) or aspirin (651) on average of 11.4 days after the onset of myocardial infarction and were followed for 6 to 59 months (mean 29 months). The results showed that aspirin in the dosage used is probably not different from oral anticoagulants in affecting mortality and morbidity after a myocardial infarction. However this study did not consider the effectiveness of either agent in comparison to no antithrombotic therapy.

In a recent study Lewis et al., (1983) published the results of a veterans administration co-operative study. This was a multicentred, double-blind, placebo controlled randomized trial of aspirin treatment (324 mg per day) for 12 weeks in 1266 men with unstable angina (625 taking aspirin and 641 placebo). Their results showed that the incidence of death or acute myocardial infarction was 51 percent lower in the

aspirin group than in the placebo group (31 patients as compared with 65). Non fatal acute myocardial infarction was 51 percent lower in the aspirin group (21 patients as compared with 44). The reduction in mortality in the aspirin group was also 51 per cent: 10 patients as compared with 21, although it was not statistically significant. The data from this study indicates that aspirin could have a protective effect against acute myocardial infarction in men with unstable angina, as well as a similar effect on mortality. The dose of aspirin used in this study (324 mg daily) is worthy of note, since there is good evidence that this dose of aspirin also produces substantial inhibition of vascular prostacyclin production (Preston et al., 1981a,b; Fitzgerald et al., 1983). This tends to suggest that the importance of prostacylin in aspirin treated patients has been overstated (Preston and Greaves, 1985). However in experiments with rabbits it was found that high doses may be thrombotic (Kelton et al., 1978) and promote atherosclerotic plaque proliferation (Stemerman 1982).

Few of the studies evaluating aspirin in patients with myocardial infarction have shown a significant reduction in mortality or coronary events but most have shown a consistent trend in favour of aspirin when the results of the studies are analysed together.

4.1.1.2. Aspirin and Cerebrovascular Disease

There is considerable evidence that platelet thromboemboli in the cerebral circulation can cause transient cerebral ischaemia and amaurosis fugax (Gunning et al., 1964; Wu and Hoak 1975). White bodies have been observed in retinal vessels during episodes of retinal ischaemia and these have subsequently been shown to be composed of platelets and fibrin (Russel, 1961). Autopsy studies have also demonstrated the presence of platelet-fibrin thrombo-emboli in cerebral and retinal blood vessels (Gunning et. al., 1964), and platelet-fibrin deposits have been demonstrated in ulcerating carotid lesions removed at endarterectomy in patients with a history of transient cerebral ischaemia.(Kelton & Hirsh , 1981b).

4.1.1.2.(a) Clinical Studies

Dyken et al., (1973) reported the results of a retrospective study which suggested that aspirin was associated with a reduced incidence of transient cerebral ischaemic attacks.

Two large cooperative studies evaluating the effects of aspirin have been reported and both are highly suggestive that aspirin is beneficial in preventing recurrent transient ischaemic attacks (TIA), stroke and death in patients with a previous history of TIA. The study by Fields et al., was reported in two parts

(1977 and 1978). The objective of the study was to assess the benefit of aspirin given in doses of 1200 mg per day to patients who had carotid transient cerebral ischaemic attacks. The first report (1977) involved patients who were treated only medically and the second (1978) described the results in patients who had reconstructive operations of the carotid artery before entry into the study. One hundred and seventy-eight patients with carotid TIA were randomly allocated to aspirin(1200 mg daily) or placebo and followed to determine the incidence of subsequent TIA, death, cerebral infarction or retinal infarction. After the first six months of follow up, when death, cerebral or retinal infarction and recurrent TIA were grouped together, there was a statistically significant reduction in the aspirin treated group compared with the placebo treated group. The difference was most marked in patients with a history of multiple TIA and in patients who had carotid lesions appropiate to the TIA symptoms. However, when the results were analysed in terms of reduction in deaths from cerebral or retinal infarction alone, there was no significant difference between aspirin and placebo. In the second report (1978), 125 patients with carotid TIA who had reconstructive operations were randomly assigned to aspirin (1200 mg per day) or placebo and followed using the same endpoints as in the first study. Aspirin had

no significant effect on the overall mortality or on the frequency of retinal or cerebral infarctions. However, when deaths which were not stroke - related were eliminated from the analysis, there was a significant reduction in the frequency of fatal and non-fatal cerebral and retinal infarctions in the aspirin treated group compared with the placebo group. However, because of the small number of patients and the short period of follow-up, the result from these studies did not prove conclusively that aspirin was effective in preventing cerebral infarction or death in patients with TIA.

The Canadian Cooperative study group (1978) reported the results of a prospective study in 585 patients with TIA, which compared four treatments. The study was a double blind trial comparing aspirin (1200 mg daily) aspirin (1200 mg daily) plus sulphinpyrazone (800 mg daily) sulphinpyrazone alone (800 mg daily) and placebo. Seventy five percent of the patients in the study had symptoms of carotid TIA and 25 per cent had vertebrobasilar TIA. The end points were continuation of TIA, stroke or death, and the results showed a statistically significant risk reduction of 19% in the aspirin treated group. A second analysis omitting TIA as an endpoint was carried out and showed a 31% risk reduction in stroke and death in the aspirin treated

group. In this study, sulphinpyrazone did not produce a statistically significant reduction for any end point and did not exhibit any synergism with or antagonism to aspirin. Of interest in this study was a striking difference in the response between males and females. There was a 48% risk reduction for stroke or death in men treated with aspirin, but no significant reduction in females. This sex difference has been found in other clinical trials in patients with cerebral vascular disease. In the studies by Kaegi et al., (1974) on arterio-venous shunts and by Harris et al., (1977) in patients undergoing hip replacement, the benefit to male patients was greater than to female patients.

Thus Aspirin has been demonstrated to be effective in reducing the incidence of stroke and death in patients with a previous history of transient cerebral ischaemia.

4.1.1.3. Aspirin and Venous Thromboembolism

Venous thrombi consist mostly of fibrin and trapped red cells but there is evidence that some start in valve pockets as aggregates of platelets which propagate with the formation of fibrin (Sevitt, 1973). In addition, platelet thrombi may form at sites of direct injury to veins, such as after trauma to the legs and to the

femoral veins during hip surgery (Clagett et al., 1975). Because of this possible role of platelets in the genesis of venous thrombosis it is feasible that antiplatelet drugs may be of value in preventing the formation of thrombi that arise principally as platelet aggregates (Turpie, 1981).

4.1.1.3.(a) Clinical Trials

Aspirin has been evaluated as an antithrombotic drug in venous thromboembolism in humans in doses of 300 to 3500 mg per day, alone and in combination with other antiplatelet drugs. In general, aspirin has not been effective in reducing the frequency of venous thrombosis in general surgical patients and the results have been mixed in patients undergoing orthopaedic procedures. In two studies in general abdomino-thoracic surgery, aspirin in doses of 600 mg and 2400 mg (O'Brien et al., 1971) and 600 mg (Medical Research Council, 1972) for the prevention of leg scan-detected venous thrombosis showed no benefit. Clagett et al., (1975) found a significant difference between the control and treated group after 1300 mg of aspirin in general surgical patients. A combination of aspirin and dipyridamole has been reported to produce a significant reduction in the incidence of postoperative venous thrombosis in general surgical patients by Rennie et al., (1976).

There have been several trials of aspirin in patients undergoing orthopaedic procedures. Salzman et al., (1971) reported the results of a comparison between aspirin, dipyridamole, warfarin and low molecular weight dextran and reported that aspirin was as effective as warfarin but that the dipyridamole was ineffective in preventing venous thrombosis. Harris et al., (1977) reported the results of a prospective controlled double-blind study of aspirin (1200 mg per day) in patients undergoing total hip replacement using venography as a diagnostic endpoint. There was a significant reduction in venous thrombosis in patients receiving aspirin, which was limited to men. Hume et al., (1977) reported the results of a non-randomized trial of aspirin and found the benefit in the treated group. Shondorf and Hey (1977) reported that intravenous aspirin in doses of 900 mg had no effect on objectively diagnosed venous thrombosis in patients undergoing elective hip surgery. A study reported by McKenna et al., (1980) in patients undergoing elective knee surgery showed that aspirin (3500mg per day) markedly reduced the frequency of objectively detected venous thrombosis.

Zekert et al., (1974; cited by Turpie,1981) reported the results of a randomized double blind trial of aspirin 1500 mg per day for the prevention of

post-operative pulmonary embolism and found a statistically significant reduction in fatal pulmonary embolism diagnosed at autopsy. Jennings et al., (1976) reported the use of aspirin (1200 mg per day)in patients undergoing total hip replacement and found no pulmonary embolism in patients in whom the expected incidence of death from pulmonary embolism was 1 to 2 per cent.

4.1.1.4 Use of Aspirin in Other Clinical Thromboembolic Conditions

While aspirin alone does not significantly decrease platelet consumption in prosthetic heart valves (as measured by platelet survival), it does significantly decrease the incidence of arterial thromboembolism in patients with prosthetic valves, when used in combinations with oral anti-coagulants (Dale et al., 1977).

Bedford and Ashford (1979) reported that aspirin significantly decreased the incidence of radial-artery thrombosis following cannulation.

Aspirin decreases thrombus formation on dialysis membranes (Lindsay et al., 1972), and when used in a dose of 165 mg/day, it reduced the formation of arteriovenous-shunt thrombosis in patients on chronic

post-operative pulmonary embolism and found a statistically significant reduction in fatal pulmonary embolism diagnosed at autopsy. Jennings et al., (1976) reported the use of aspirin (1200 mg per day)in patients undergoing total hip replacement and found no pulmonary embolism in patients in whom the expected incidence of death from pulmonary embolism was 1 to 2 per cent.

4.1.1.4 Use of Aspirin in Other Clinical Thromboembolic Conditions

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Bedford and Ashford (1979) reported that aspirin significantly decreased the incidence of radial-artery thrombosis following cannulation.

Aspirin decreases thrombus formation on dialysis membranes (Lindsay et al., 1972), and when used in a dose of 165 mg/day, it reduced the formation of arteriovenous-shunt thrombosis in patients on chronic

haemodialysis (Harter et al., 1979).

Aspirin has been reported to decrease thromboembolic complications (cerebral embolism) following coronary angiography (Storstein et al., 1977). Aspirin has also been demonstrated to be effective in preventing the small vessel ischaemic episodes in the toes and fingers in patients with essential thrombocythemia (Preston et al., 1974).

Aspirin has been demonstrated to be effective in reducing the incidence of completed stroke and death in patients with transient cerebral ischaemic episodes and in reducing the incidence of silastic-arteriovenous-shunt thrombosis in patients undergoing chronic haemodialysis. It has also been reported to reduced the frequency of postoperative venous thrombosis following hip surgery. When combined with oral anticoagulants aspirin has been shown to be effective in reducing the frequency of systemic embolism in patients with prosthetic heart valves. Evidence from a number of studies also suggest that aspirin might be efficacious in decreasing sudden death following myocardial infarction, but the results to date have not been definite. (Kelton and Hirsh, 1981b).

The difficulties in assessing the benefits of aspirin treatment became clear on examination of recent

analyses of clinical trials of platelet inhibitors on vascular disease (see above). The doses used in clinical trials have varied from 324-1500 mg per day given in 1-4 equal doses. The logic of administering these doses has been questioned (Marcus, 1977) and much lower doses have been advocated (Szceklik et al., 1979; Patrono et al., 1980; Masotti et al., 1979; Mitchell, 1980; Hanley et al., 1982; Preston et al., 1982; Svensson and Samuelsson, 1983; Boysen et al., 1984; Jakubowski et al., 1985.

The optimal dosage regimen remains a key area for investigation. The optimal aspirin dose is one that achieves inhibition of platelet prostaglandin synthesis, but does not interfere with vessel wall prostacyclin formation.

Since prostacyclin production can resume between doses of aspirin (de Gaetano et al., 1982; Marcus 1983), the administration of low doses of the drug could prevent the inhibition of total suppression of PGI_2 while suppressing TXA_2 and thus shifting the TXA_2: PGI_2 balance towards PGI_2. Unfortunately, studies with cultured endothelial cells (Jaffe and Weksler, 1979) laboratory animals (Villa et al ., 1979) and man

(Masotti et al., 1979; Preston et al., 1981a,b; Hanley et al., 1981), have not completely dissociated the inhibitory effect of aspirin on platelets from that on vascular cells. Moreover work in rats (Villa et al., 1979) and in rabbits (Buchanan et al., 1980) has shown that the duration of the aspirin effect may vary widely in different types of blood vessels.

Considerable efforts have been made to find a so called "therapeutic window" i.e. the lowest possible dose that effectively blocks the pro-aggregatory TXA_2 while sparing the synthesis of anti-aggregatory prostacyclin as much as possible. A wide range of doses and dose regimens have been suggested . The lowest dose being 20 mg per day (Patrignani et al., 1982). This dose produced virtually complete inhibition of platelet TXB_2 synthesis without affecting the production of prostacyclin when measured as the urinary excretion of 6-Keto-PGF_1 . This was observed among healthy volunteers (Patrignani et al., 1982) and cardiac patients (De Caterina et al 1982). However, in another study (Boysen et al., 1982), platelet aggregation was investigated in 42 patients with ischaemic cerebrovascular disease given low doses of aspirin. The aggregation was inhibited in only 78% of those given 50 mg aspirin per day and in two of them 125 mg aspirin per day was required to achieve complete

inhibition. Although Patrignani et al., (1982) showed that 20 mg aspirin per day inhibited TXB_2 synthesis with no effect on urinary 6-Keto PGF_1 , they did not monitor platelet aggregation, so it remains to be established whether inhibition of thromboxane generation in this way suppresses platelet aggregation. The TXB_2 and 6-Keto-PGF_1 measurements are valuable but must be supplemented by and correlated with detailed studies of in vitro platelet aggregation and bleeding times before and during aspirin administration. Otherwise, no conclusions can be drawn about the effect of therapy on in vivo platelet responsiveness.

The duration of the aspirin effect and therefore the frequency of dosage has also not been established. The recovery of cyclooxygenase activity in circulating platelets follows a time course corresponding approximately to the mean life span of platelets (Burch et al., 1978; Roth et al., 1975; Stuart et al., 1975; Roncucci et al., 1977). As new platelets enter the circulation the proportion of normal to inhibited platelets will rise steadily but it is not known whether or not the return of effective platelet function follows a similar pattern.

Cerskus et al., (1980) have suggested on the basis of experiments in rabbits that an admixture of 10% normal

platelets to a population of aspirin treated platelet can restore normal or near normal platelet function. Assuming a mean platelet life span of 10 days effective platelet function could return within 24 hours of a dose of aspirin. If a higher proportion of normal platelets is needed to achieve normal function the duration of effect would be correspondingly longer, perhaps many days. Some workers (Jaffe and Weksler, 1979; Amezcua et al., 1979; Marcus, 1983) have suggested that aspirin should be given every 24 hours while others (Masotti et al., 1979; Patrono et al., 1980; Szceklik et al., 1979) have advocated a dose every third or fourth day since this might cause the least possible inhibition of endothelial cell PGI_2 production and yet could maintain adequate suppression of platelet activity. However, if the observation of Cerskus et al., (1980) applies equally to human subjects the duration of effective aspirin induced platelet suppression may be as short as 24 hours or less. In that event more widely spaced aspirin doses may be inadequate to achieve an antithrombotic action.

This study was undertaken to determine 1) the effect of normal platelets on aspirin inhibited platelets in human subjects and to assess the relevance of this effect to dosage frequency when aspirin is used to prevent thrombosis and, 2) The effect of 20 mg aspirin on platelet aggregation.

4.2 Methods

4.2.1 Ex Vivo Studies

Seven normal subjects were each given 300mg aspirin (single dose) orally. Blood samples were collected with 1 part 3.8% sodium citrate to 9 parts blood before the aspirin dose, and at daily intervals for a further 10 days. Platelet rich plasma (PRP) was prepared from the samples and the platelet count of the PRP was adjusted to $250 \pm 10 \times 10^9$ per litre.

4.2.1.(1) Platelet aggregation

Platelet aggregation studies were carried out as described -sect.3.3, with specific attention to the following : the concentration of agonist chosen for analysis was that which demonstrated the greatest difference between the pre-treatment aggregability and the maximum effect of the aspirin dose. The concentrations used were: ADP 1.6 or 2.1. uM, adrenalin 1.3, 2.6 or 3.9 uM, collagen 1 or 2 ug/ml and Arachidonic acid 0.5, 0.6 or 1 mM

For each agonist the height of the maximum aggregation response above the baseline at the chosen concentration was measured in millimeters before and on successive days after the aspirin dose. Each post aspirin observation was expressed as a percentage of the pre aspirin response. In the case of ADP only the height

of the secondary wave was measured. Many of the post aspirin responses to ADP showed a primary wave followed by disaggregation. When this occurred the response was recorded as zero. For the other three agonists the maximum response (whether a single wave or two waves of aggregation) was measured.

4.2.1(2) Malonyldialdehyde (MDA) Production

MDA production from 0.2ml of PRP was measured as described in section 3.4. Results were expressed as nmoles of MDA Per 10^8 platelets produced after stimulation with 2 mM AA.

4.2.1(3) Platelet survival

Platelet survival in four normal subjects was measured using Indium ^{111} labelled platelets by the method of Thakur et al., (1976) as modified by Heyns et al., (1980) as described in section 3.8.

4.2.2. In Vitro Studies

Thirteen volunteers were given 600 mg and nine received 150 mg aspirin (single dose) orally. Blood was collected (1 part 3.8% sodium citrate to 9 parts blood) before and two hours after the aspirin dose. PRP ($250\pm10 \times 10^9$ platelets per litre) was prepared from both samples immediately after collection. The PRP from the first sample was kept at room temperature for 2 hours by which time PRP from the second specimen was prepared.

4.2.2 (1) Platelet Aggregation

Aggregation studies were carried out on both preparations and on mixtures of the two. The pre aspirin PRP was tested for sensitivity to each of the aggregating agonists to be tested and the least concentration required to give maximum aggregation in each case was used for all subsequent aggregation studies on the pre and post aspirin PRP mixtures. The agonists used and the concentrations selected were the same as before. The two PRP preparations were mixed to give the following proportions of normal to post-aspirin platelets: 1:9, 2:8, 3:7, 4:6, 5:5 and 6:4 corresponding to 10, 20, 30, 40, 50 and 60% normal platelets in the mixtures.

The possibility existed that circulating plasma levels of aspirin (after an aspirin dose) might inhibit the pre aspirinised platelets in the *in vitro* mixing model. To explore this the following control experiment was performed. A volunteer was given 600 mg aspirin orally. Forty ml citrated blood was collected before and 1 hour after the aspirin dose. PRP and PPP was prepared from both blood samples. The following plasma mixtures were prepared for aggregation studies:

i) Sample 1 - pre aspirin PRP. diluted with pre aspirin PPP.

ii) Sample 2 - post aspirin PRP diluted with post aspirin PPP, and

iii) Sample 3 - pre aspirin PRP diluted with post aspirin PPP

All samples had a platelet count of 250 $\pm$ 10 x 10^9 per litre. Sample 1, reflects normal platelet aggregation responses, sample 2 the effect of aspirin on platelet aggregation, and sample 3 the effect of circulating plasma aspirin on normal platelets.

4.2.2.2. Thromboxane B_2 Production

After aggregations had occurred the specimens were centrifuged for 30 minutes at 1800g to produce platelet poor plasma (PPP). Thromboxane B_2 in the PPP was measured as described in section 3.5.

4.2.3. Effects of 20mg aspirin

Nine normal subjects (mean age = 21 years, range = 20 - 22 years, 7 males, 2 females - all medical students were given 20mg of aspirin daily for 28 days. To achieve this 20mg analytical grade acetyl salicyclic acid was weighed into glass tubes. After swallowing the powder the subjects rinsed any remaining drug from the tubes with tap water, which they swallowed. All subjects had not taken aspirin or any other drug known to affect platelet function for at least 2 weeks prior to the experiment. They were also instructed to fast and not to smoke on the morning of each test. Blood samples

were collected before commencing the aspirin therapy and at two and four weeks later.

4.2.3.1. Platelet Aggregation

Blood samples were collected with 1 part 3.8% sodium citrate to 9 parts of blood. Platelet rich plasma (PRP) was prepared from the samples and the platelet count of the PRP was adjusted to 250 $\pm$ 10 x 10^9 per litre. Platelet aggregation and quantitation was determined as described above (section 4.2.1.1).

4.2.3.2. Thromboxane B_2 Production

After aggregation had occured the specimens were centrifuged for 30 minutes at 1800 g to produce platelet poor plasma (PPP). Thromboxane B_2 in the PPP was measured by radioimmuno assay as described in section 3.5.

4.2.3.3. Bleeding Times

Bleeding times were measured using a modification of the Ivy method. A sphygmomanometer cuff was maintained at a pressure of 40mm Hg and the skin of the forearm was punctured using a Simplate II Bleeding Time Device (General Diagnostics, Morris Plains, N.J. 07950).

4.3. RESULTS

4.3.1. Ex Vivo Studies

4.3.1(1) Platelet aggregation and MDA Production

The expected depression of platelet aggregation (figs 4.1 and 4.4) and fall in MDA production (Fig. 4.5) was present at 24 hours after the 300 mg oral dose of aspirin. Aggregation in response to AA was reduced to zero and the second wave of aggregation following ADP stimulation disappeared in all cases. (Fig. 4.6 and Fig. 4.7 respectively). The responses to adrenalin and ADP were reduced to very low levels but did not disappear entirely except in two cases in which the ADP response disappeared on day 1 only.

Definite evidence of partial recovery from the aspirin inhibition was present in all cases on day 4, by day 6 aggregation was above 50% of the base line value in most cases and by day 7 recovery was complete or nearly so in all cases except for the response to ADP in three cases. On day 3 there was some evidence of recovery of aggregation responses in only one case but MDA production showed some increase in all cases tested (Figure 4.5).

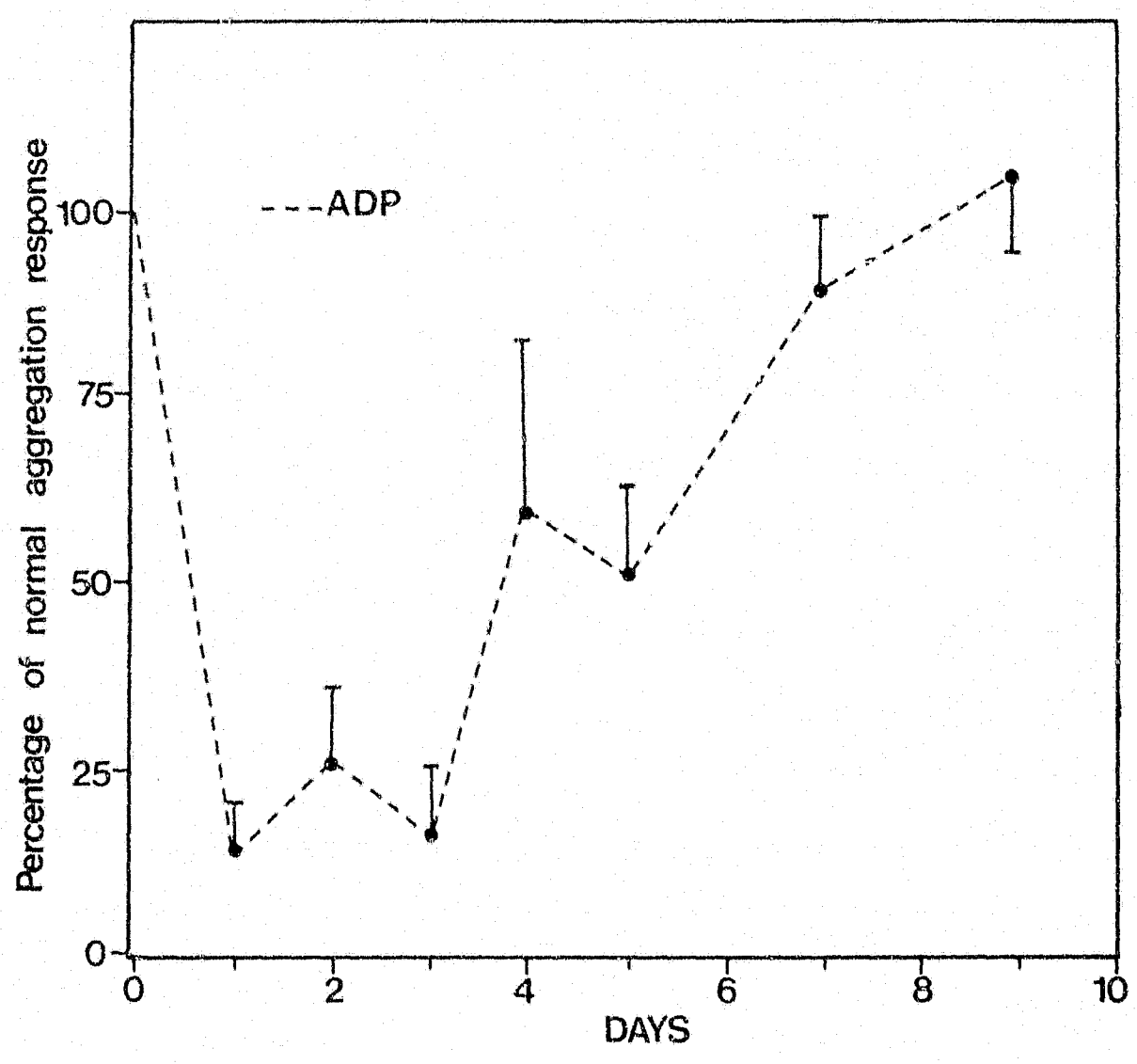


Fig. 4.1 Effects of 300 mg Aspirin on aggregation response to ADP in seven normal subjects. Aggregation response calculated as percentage of pre aspirin response (see methods). Mean ± SEM are shown.

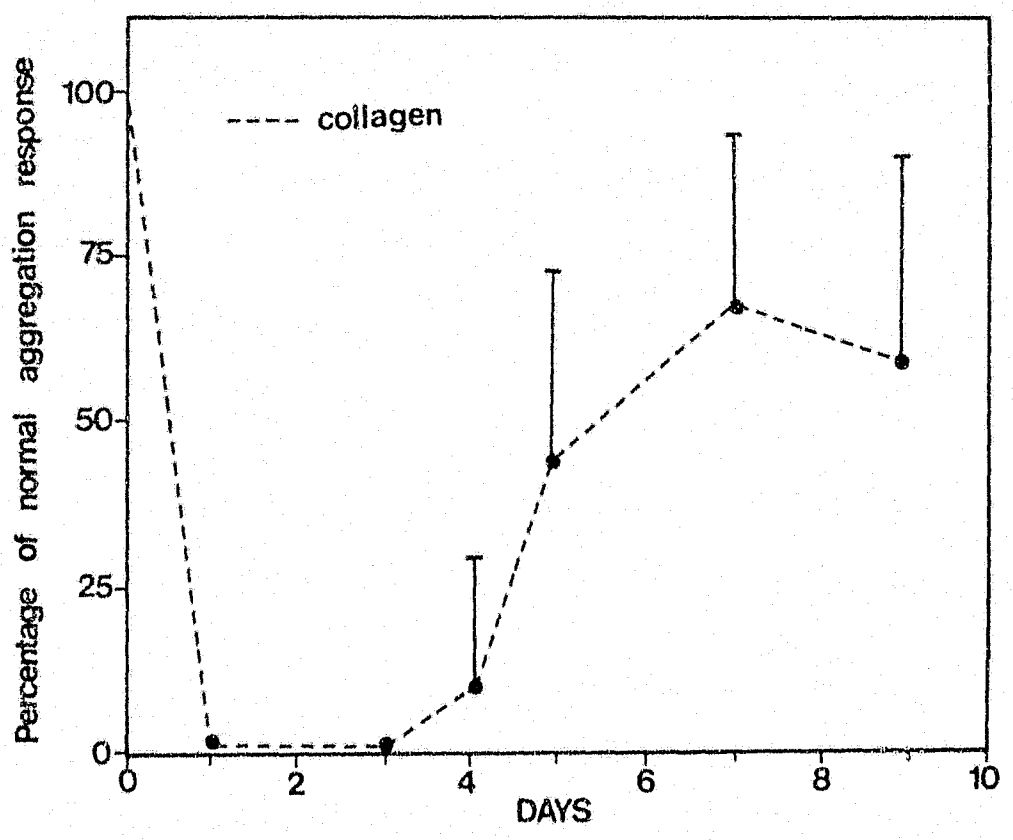


Fig. 4.2. Effects of 300 mg Aspirin on aggregation response to collagen in seven normal subjects. Aggregation responses calculated as a percentage of pre aspirin response (see methods). Mean ± SEM are shown.

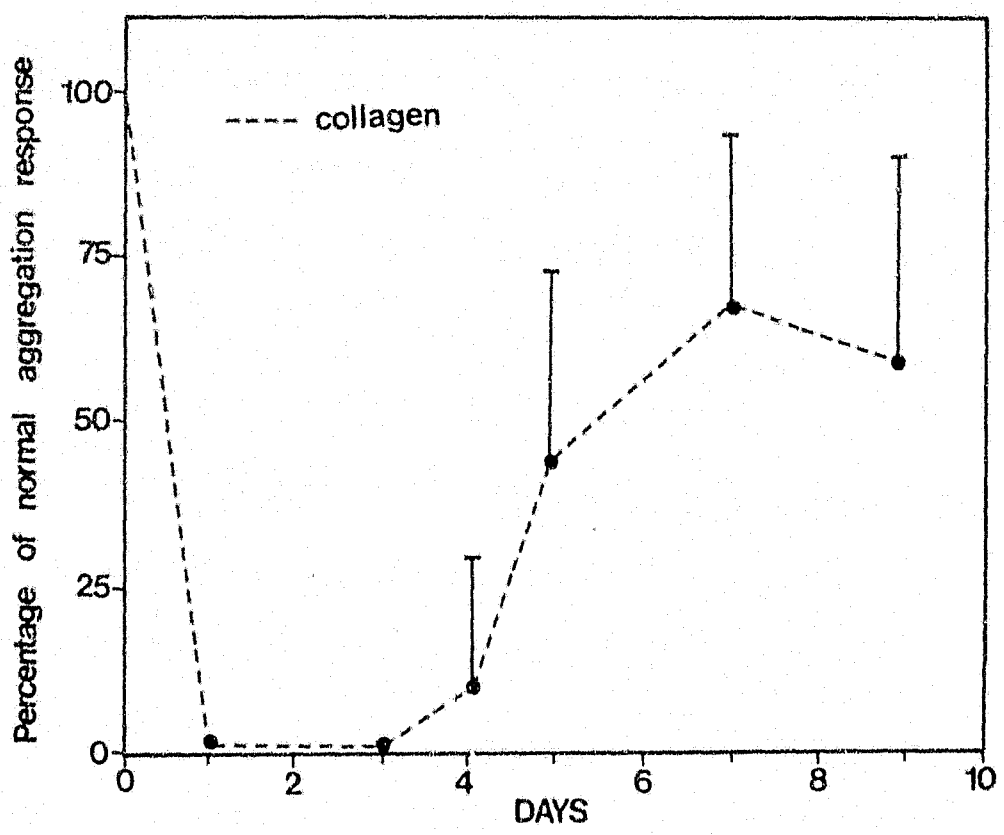


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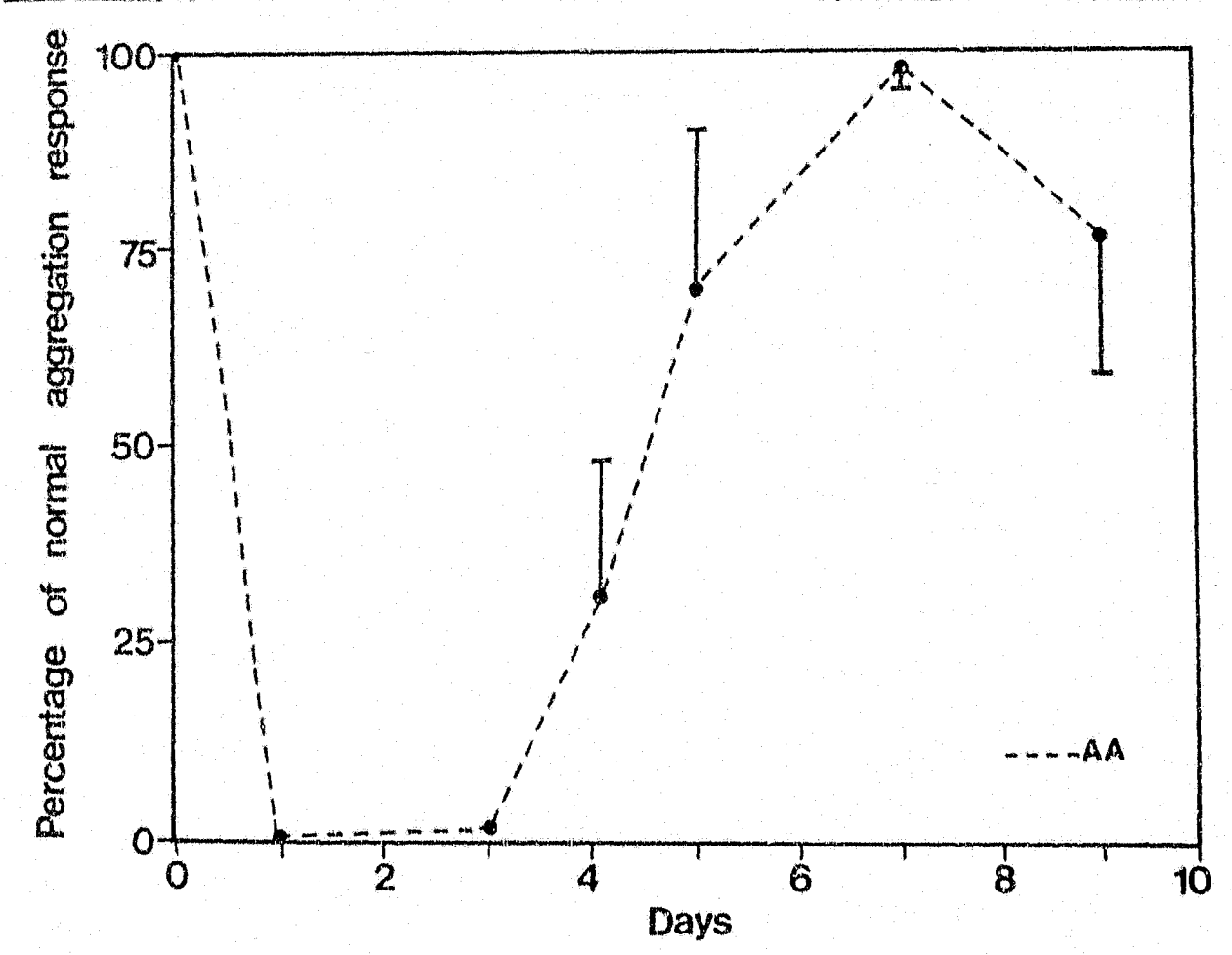


Fig. 4.3. Effects of 300 mg Aspirin on aggregation response to arachidonic acid (AA) in seven normal subjects. Aggregation responses calculated as a percentage of pre aspirin response (see methods). Mean ± SEM are shown.

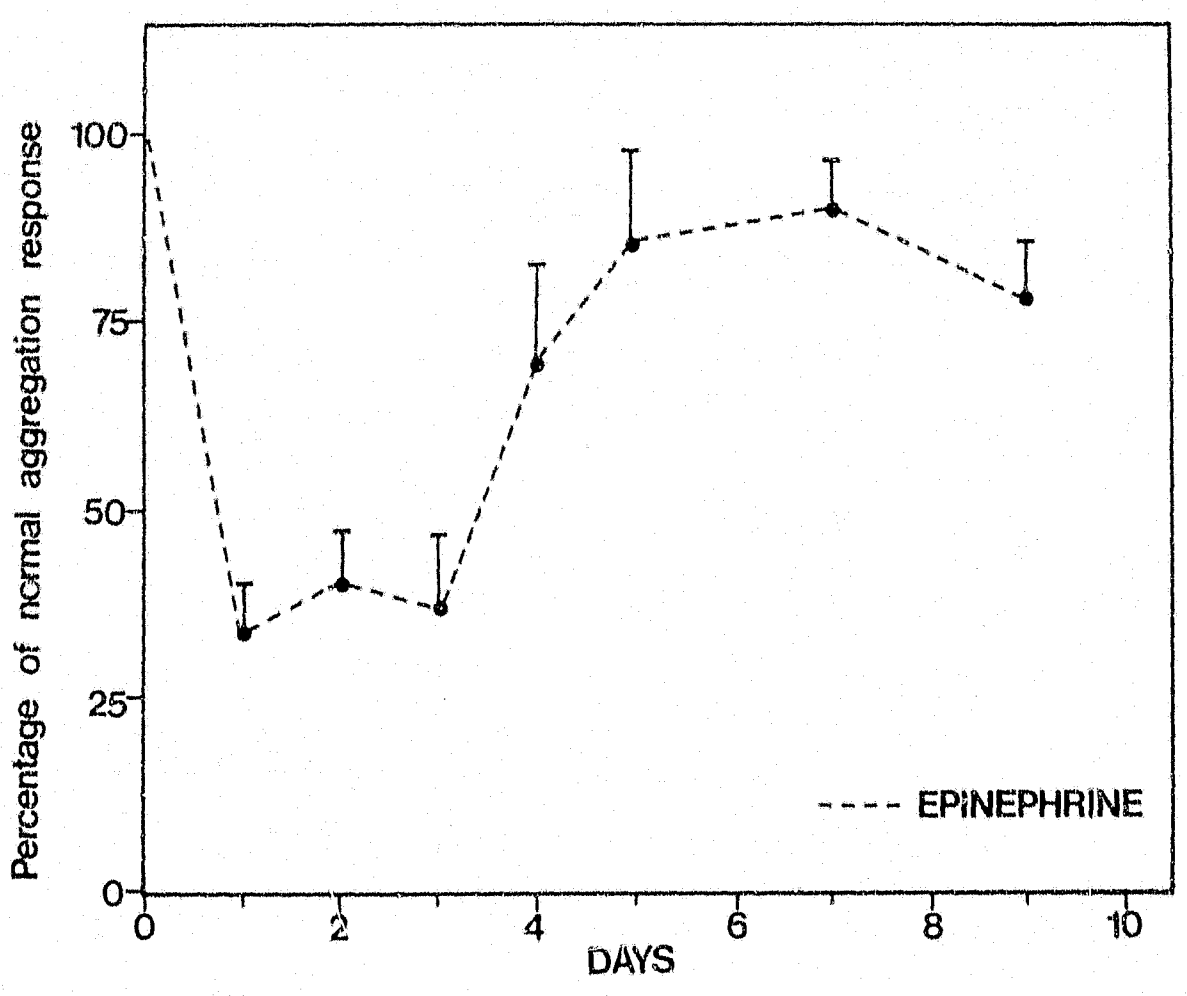


Fig. 4.4. Effects of 300 mg Aspirin on aggregation in response to epinephrine in seven normal subjects. Aggregation responses calculated as a percentage of pre aspirin response (see methods). Mean ± SEM are shown.

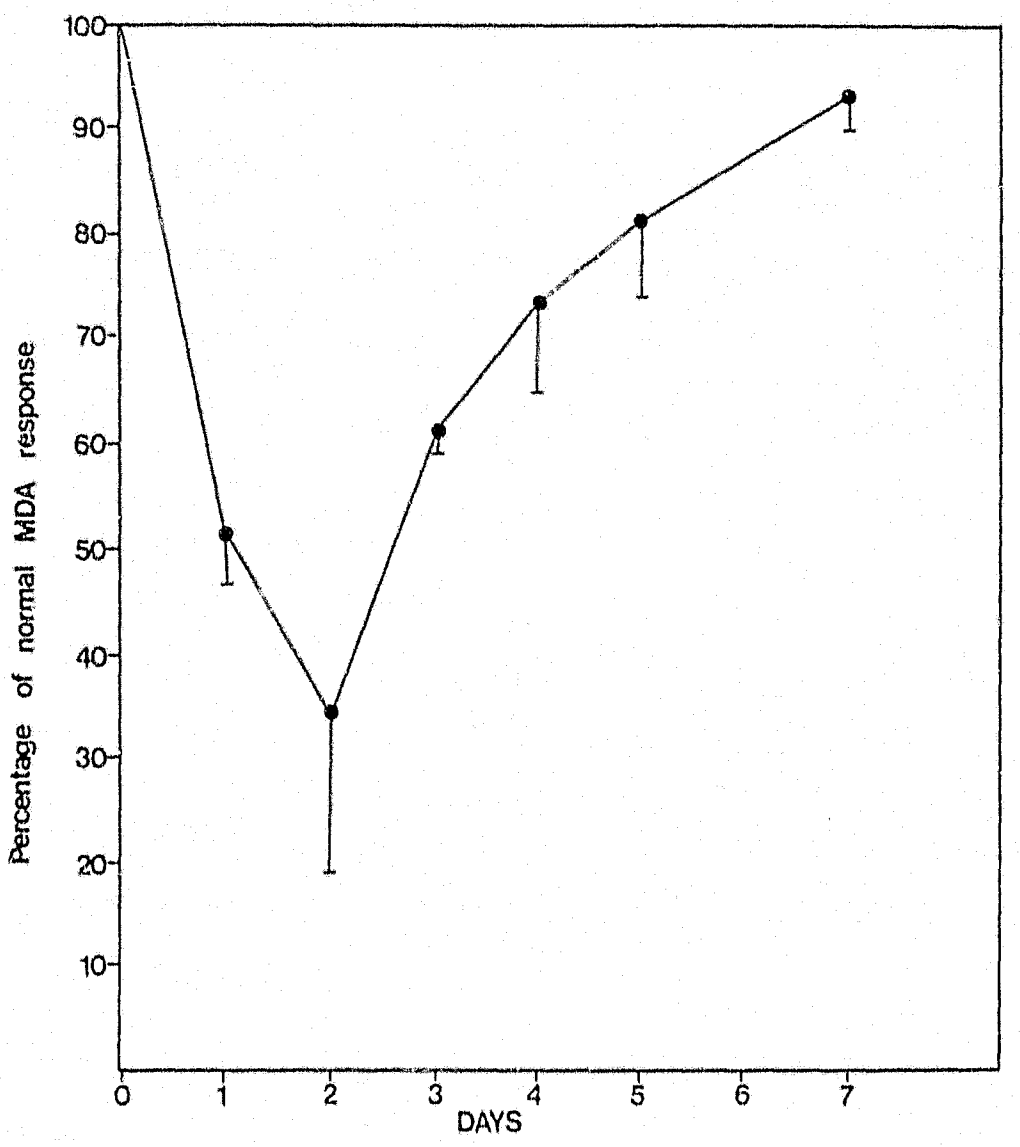


Fig 4.5

Suppression and recovery of MDA production after a single dose of 300 mg of aspirin.

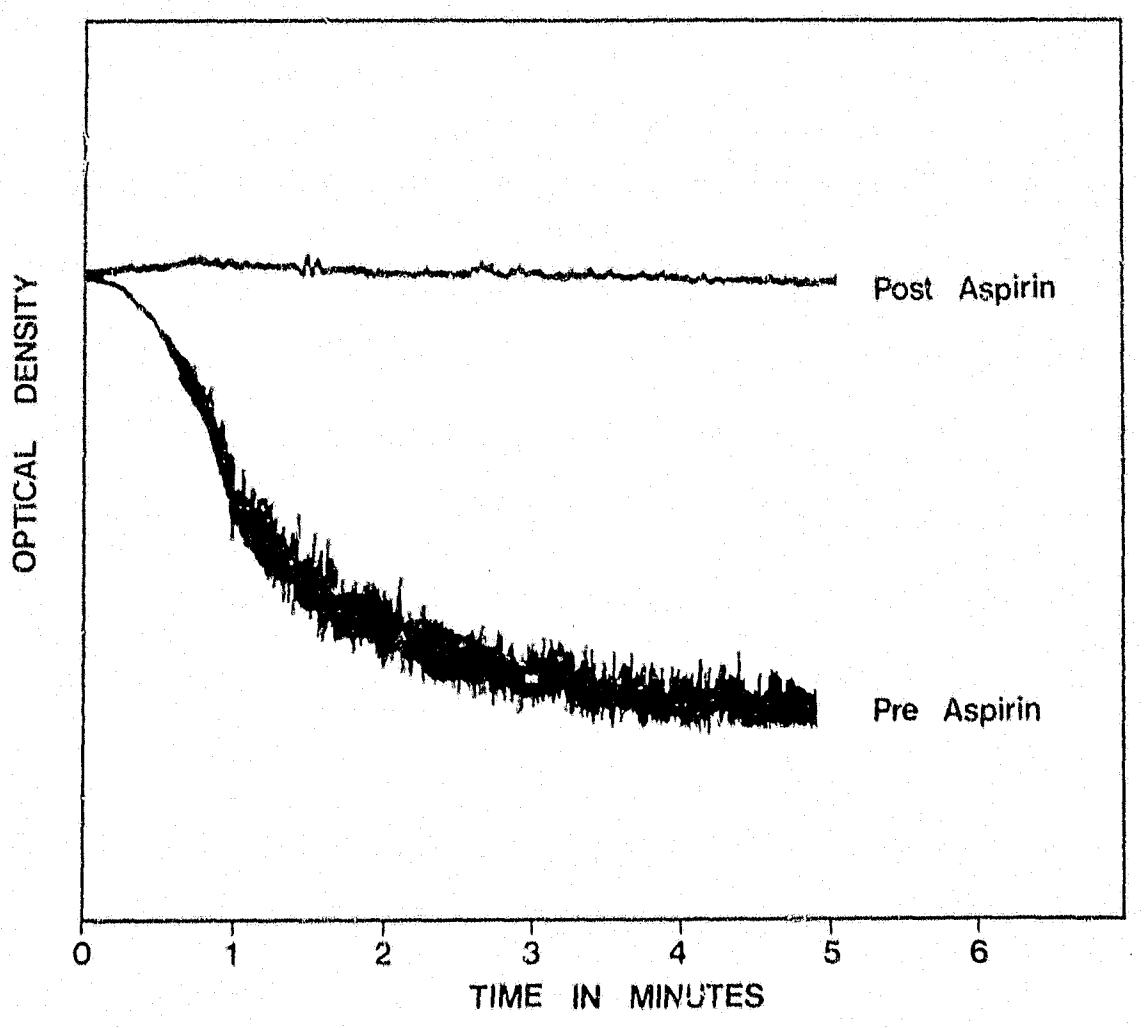


Fig 4.6 Arachidonic Acid irduced aggregation reduced to zero after aspirin ingestion.

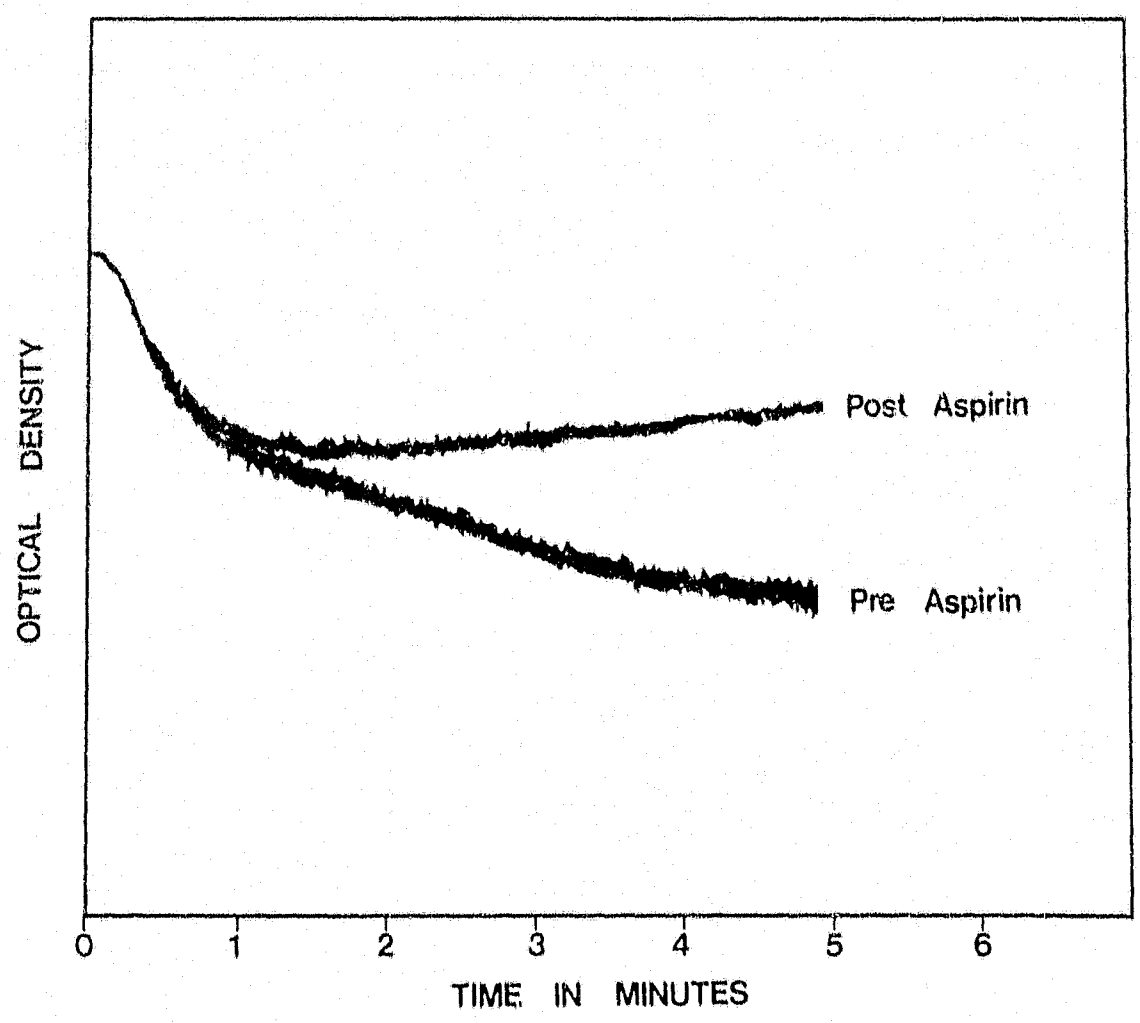


Fig 4.7 Dissapearance of the second wave of ADP stimulated aggregation after aspirin ingestion.

4.3.1(2) Platelet survival studies

In four of the subjects studied mean platelet life span was determined several weeks prior to the aspirin experiment. In all four subjects platelet aggregation returned to normal (defined as more than 75% of full aggregation) in two to seven days less than the mean platelet life span. (Table 4.1)

Table 4.1 A comparison of mean platelet lifespan determined by the ^{111}Indium labelled platelet method and the time required for aggregation responses to return to normal after aspirin (300 mg) ingestion in four normal subjects

Subjects	Mean platelet life span(Days)	Days required for aggregation to return to normal after ASA ingestion			
		ADP	Adrenalin	Collagen	AA
1	10.8	8	5	4	5
2	11.0	8	4	7	4
3	9.6	5	4	4	5
4	10.2	6	6	4	6
Mean	10.4	6.75	4.75	4.75	5.0

4.3.2 In vitro studies

4.3.2.1.(a) Platelet aggregation.

Results obtained from subjects given 150 mg aspirin were not significantly different from those given 600 mg ($p > 0.05$, Fishers exact probabilities calculated from 2 x 2 table). All the results were therefore pooled. Mixtures of normal and aspirin inhibited platelets were tested for aggregation responses. A representative experiment showing the effect of increasing the proportion of normal platelets in the mixture is shown in Figs 4.8 - 4.11 for each of the agonists. Fig 4.12 shows the percentage of normal platelets required to produce full and partial (defined as more than 75% and 25% of full aggregation respectively) aggregation in 22 normal subjects.

Table 4.2 shows that the mean values for the percentage of normal platelets required to produce full aggregation in a mixture of normal and aspirin treated platelets varied between 25 and 53% for the different agonists. In the case of ADP full aggregation was restored by 10% of normal platelets in 4 cases and 20% in seven cases. Aggregation to adrenalin was restored fully in three cases by 10% of normal platelets and by 20% in three other cases. In most instances the proportion of normal platelets necessary was much higher.

Partial restoration of aggregation, was produced by a significantly lower proportion of normal platelets. With 1[illegible] normal platelets partial restoration of aggregation was found in 13 cases after stimulation with ADP, 8 cases with Adrenalin, and 1 case with collagen and AA. With 20% normal platelets an additional 5 cases with ADP, 5 with adrenalin, 4 with collagen and 4 with AA achieved partial restoration of function (fig 4.12).

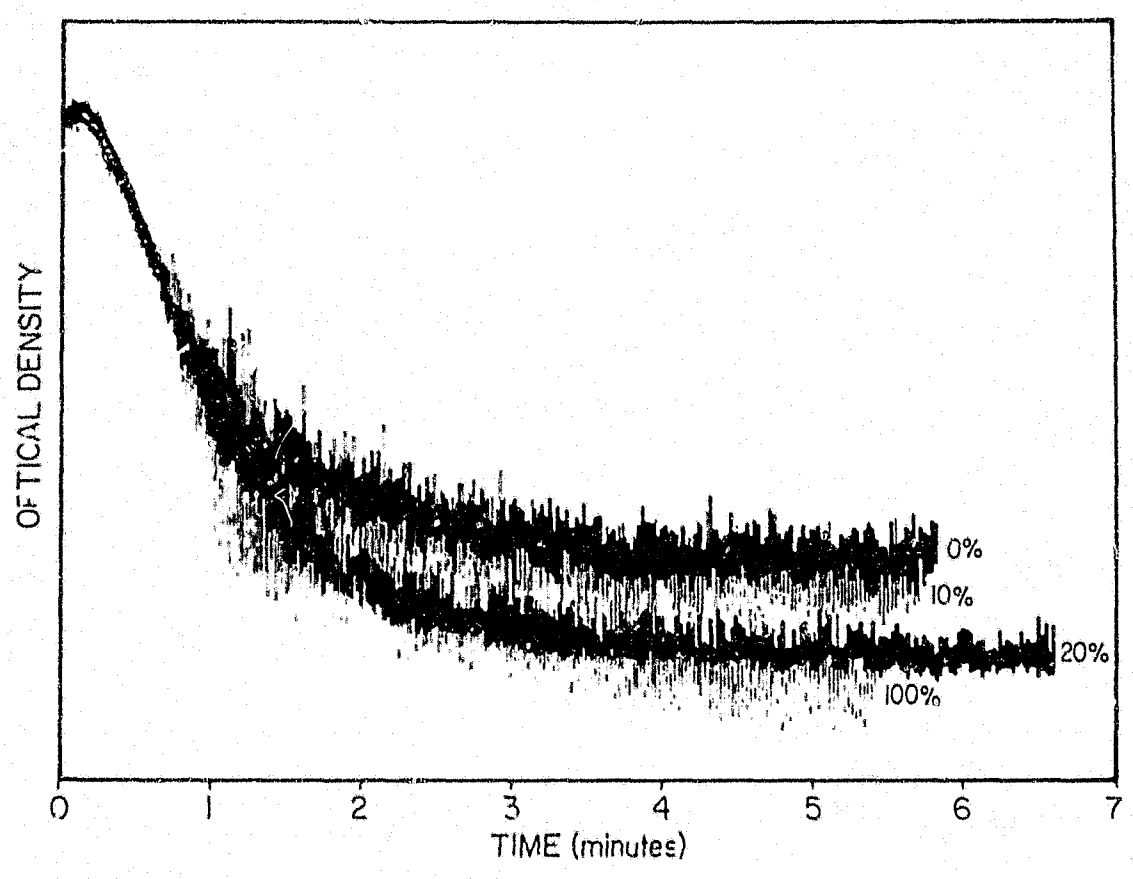


Fig 4.8 Aggregation responses to ADP in mixtures of normal and aspirin inhibited platelets from one normal subject. The figures shown next to each tracing represent the percentage of normal platelets in the mixture.

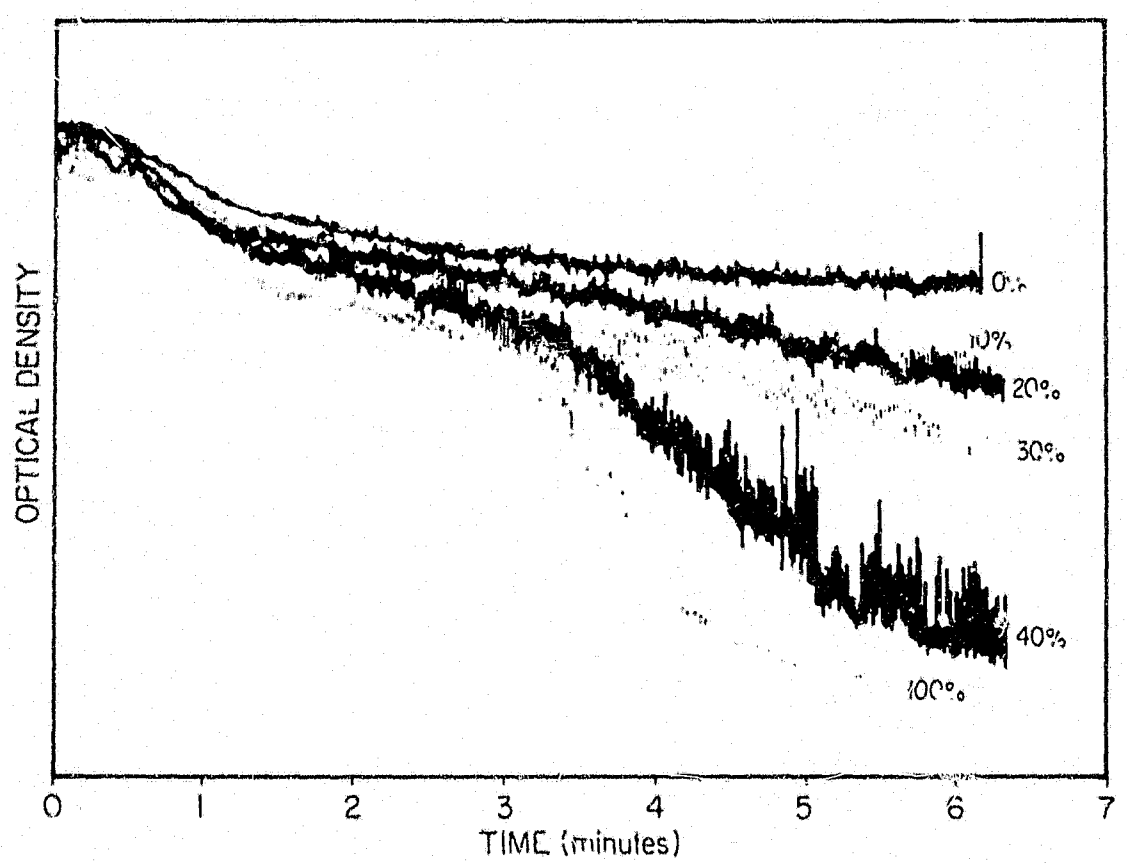


Fig 4.9 Aggregation responses to adrenalin in mixtures of normal and Aspirin inhibited platelets from one normal subject. The figures shown next to each tracing represent the percentage of normal platelets in the mixture.

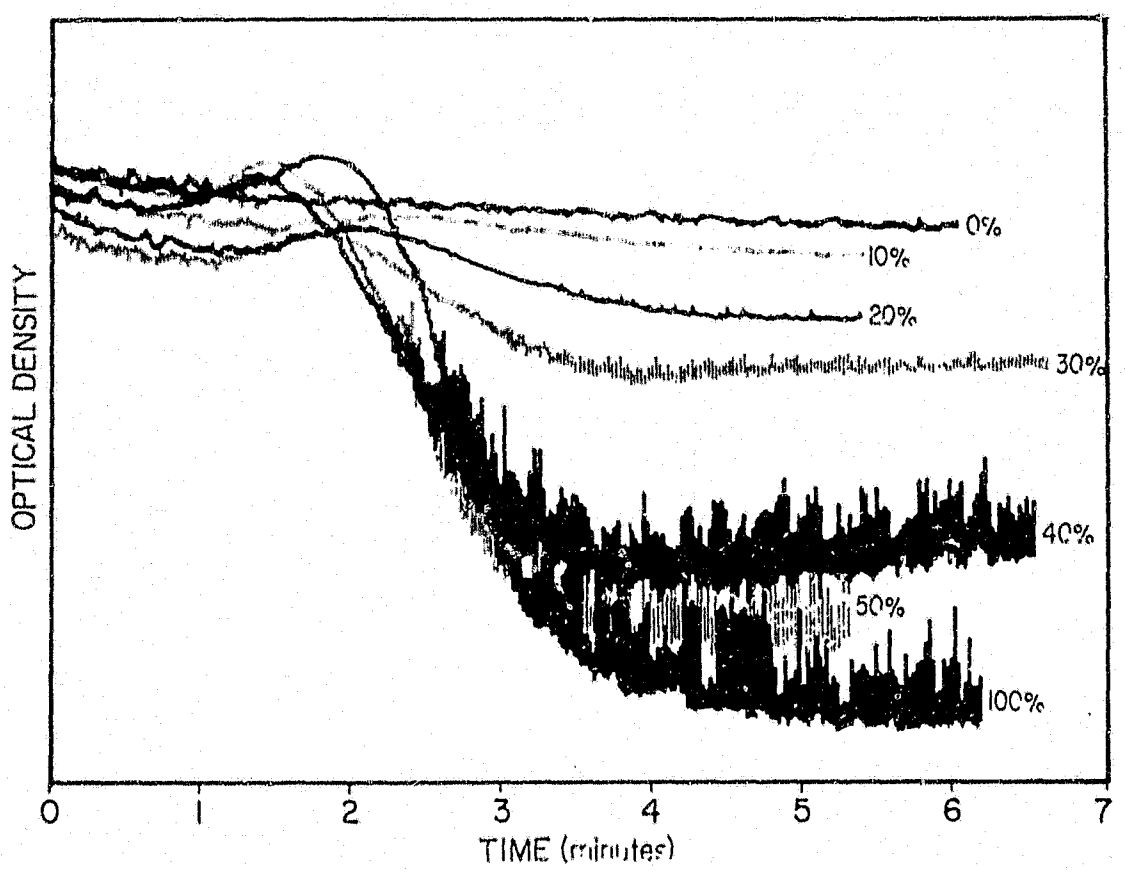


Fig 4.10 Aggregation responses to collagen in mixtures of normal and aspirin inhibited platelets from one normal subject. The figures shown next to each tracing represent the percentage of normal platelets in the mixture.

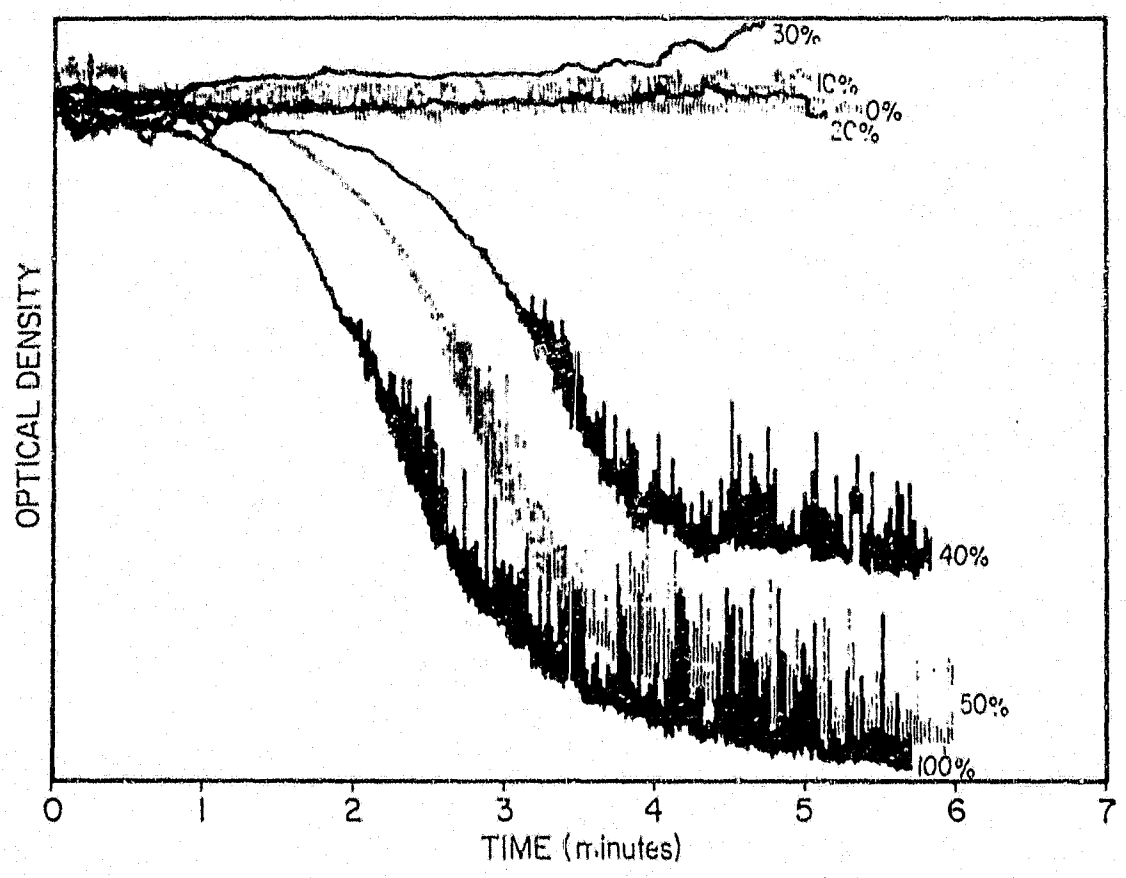


Fig 4.11. Aggregation responses to arachidonic acid in mixtures of normal and aspirin inhibited platelets from one normal subject. The figures shown next to each tracing represent the percentage of normal platelets in the mixture.

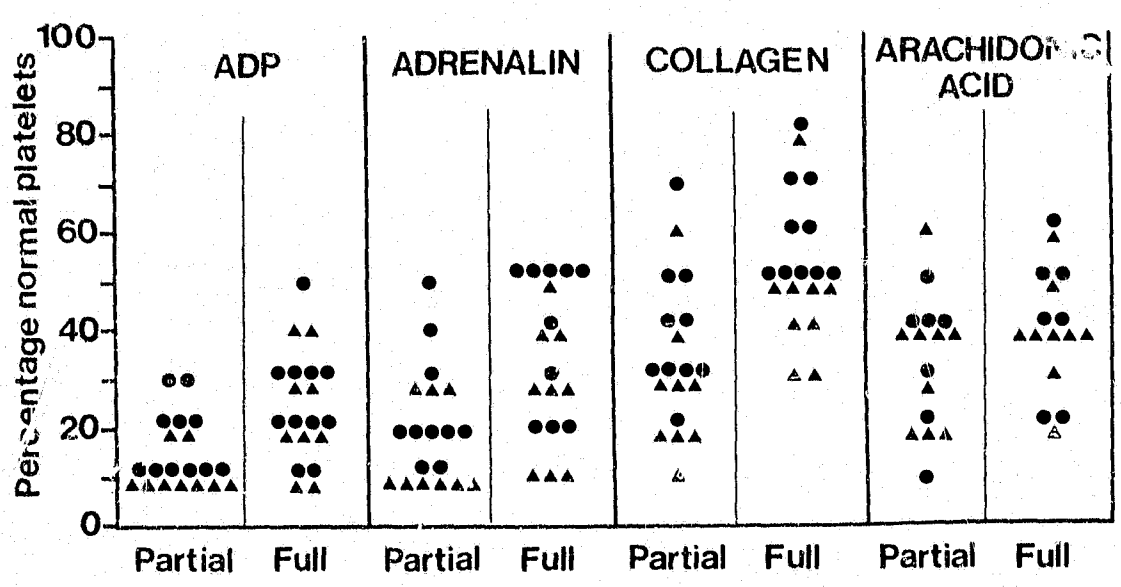


Fig 4.12. Percentage of normal platelets required to restore platelet function in mixtures of normal and aspirin inhibited platelets in 22 normal subjects ●= subjects given 600 mg Aspirin ▲= subjects given 150 mg aspirin. Full aggregation was defined as >75% and partial aggregation as >25% of the response of normal (uninhibited) platelets.

Table 4.2. Percentage of normal platelets required to restore full and partial aggregation responses to mixtures of normal and aspirin inhibited platelets.

	ADP		Adrenalin		Collagen		AA	
	P	F	P	F	P	F	P	F
Mean	15	25	21	33	35	53	34	40
S.D.	7	11	12	15	15	14	14	13
N	20		19		19		16	

Full aggregation was defined as > 75% and partial as > 25% of the response of normal (uninhibited) platelets. N = number of subjects tested. P=Partial. F=Full. Results are the mean values derived from one experiment on each of the six subjects.

4.3.2.1.(b) Thromboxane B_2 production.

Thromboxane production after stimulation with each of four agonists was measured in normal platelets. (Table 4.3) and in mixtures of normal and inhibited platelets (Figs 4.13-4.16). With all four of the agonists TxB_2 production expressed as a percentage of the production in uninhibited platelets (fig. 4.13-4.16) approximated the percentage of normal platelets in the mixture. Actually, in all 4 cases restoration of aggregation seems greater then restoration of TXB_2. The results suggested that normal platelets were not responsible for inducing production of TxB_2 by the inhibited platelets and that normal aggregation occured with considerably less than maximal TXB_2 production.

Table 4.3 Thromboxane B_2 production after stimulation by the 4 agonists.

Aggregating Agent	Concentration*	Thromboxane B_2 Production p Moles/ml of Plasma	
		Mean	S.D.
Arachidonic acid	0.5 - 1.0 mM	210.3	71.9
Collagen	2 - 4 ug/ml	11.8	3.5
ADP	2.1 - 10.6 uM	4.2	2.1
Adrenalin	1.3 - 5.5 uM	5.9	2.4

*The concentration of aggregating agent used for each reagent was the minimum required to produce maximal aggregation and was determined by experiment for each of the six subjects and for each agonist.

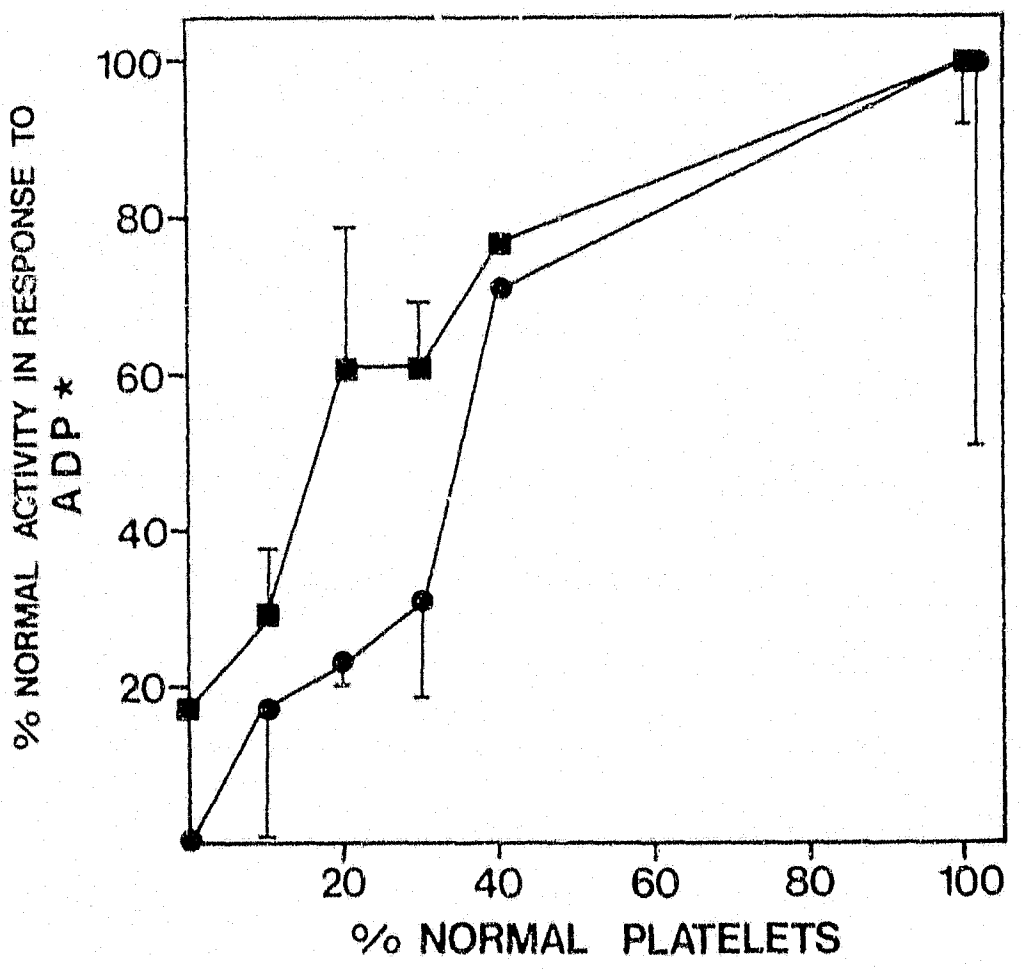


Fig 4.13. Aggregation responses and TXB_2 production in mixtures of normal and aspirin inhibited platelets in response to ADP. Means ± S.D. derived from 13 normal subjects are shown. All results are calculated as the percentage of the aggregation response and of TxB_2 production in normal platelets.

■ - Aggregation

● - Thromboxane B_2

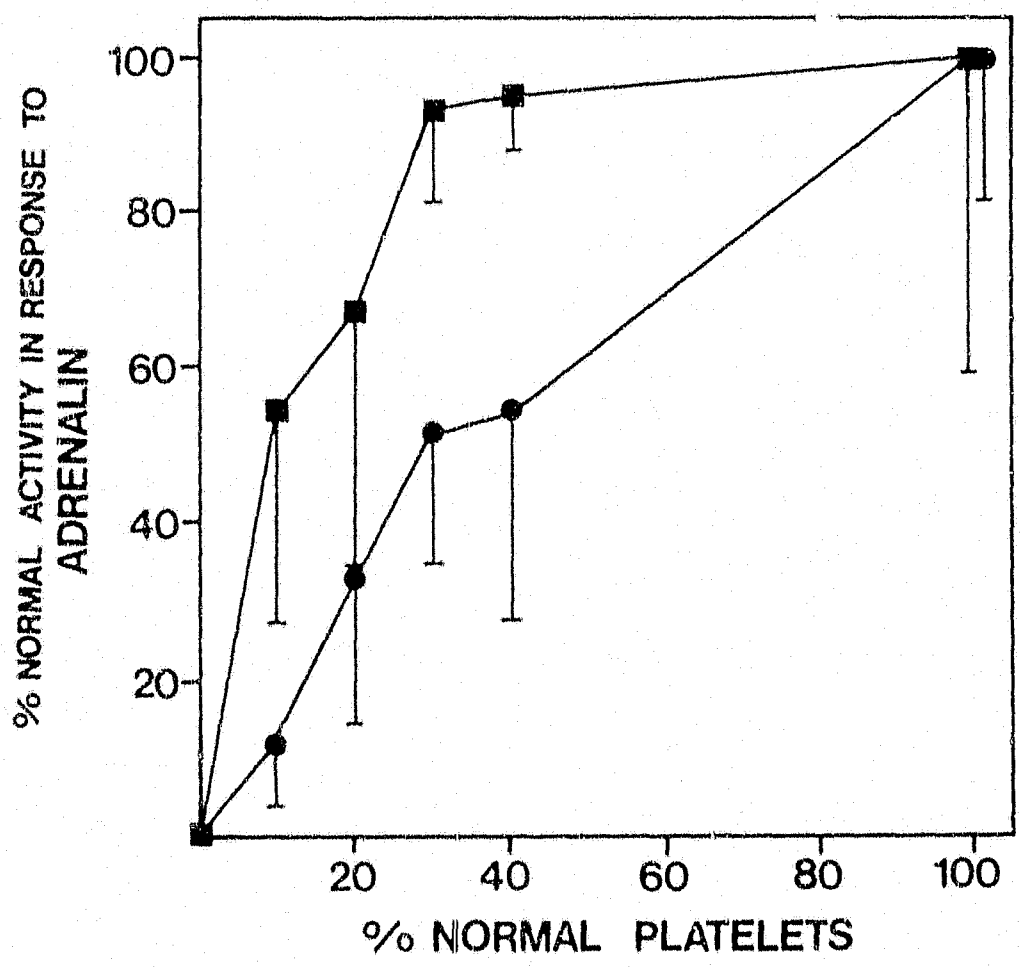


Fig. 4.14. Aggregation responses and TXB_2 production in mixtures of normal and aspirin inhibited platelets in response to adrenalin. Means ± S.D. derived from 13 normal subjects are shown. All results are calculated as the percentage of the aggregation response and of TxB_2 production in normal platelets.

■ - Aggregation

● - Thromboxane B_2

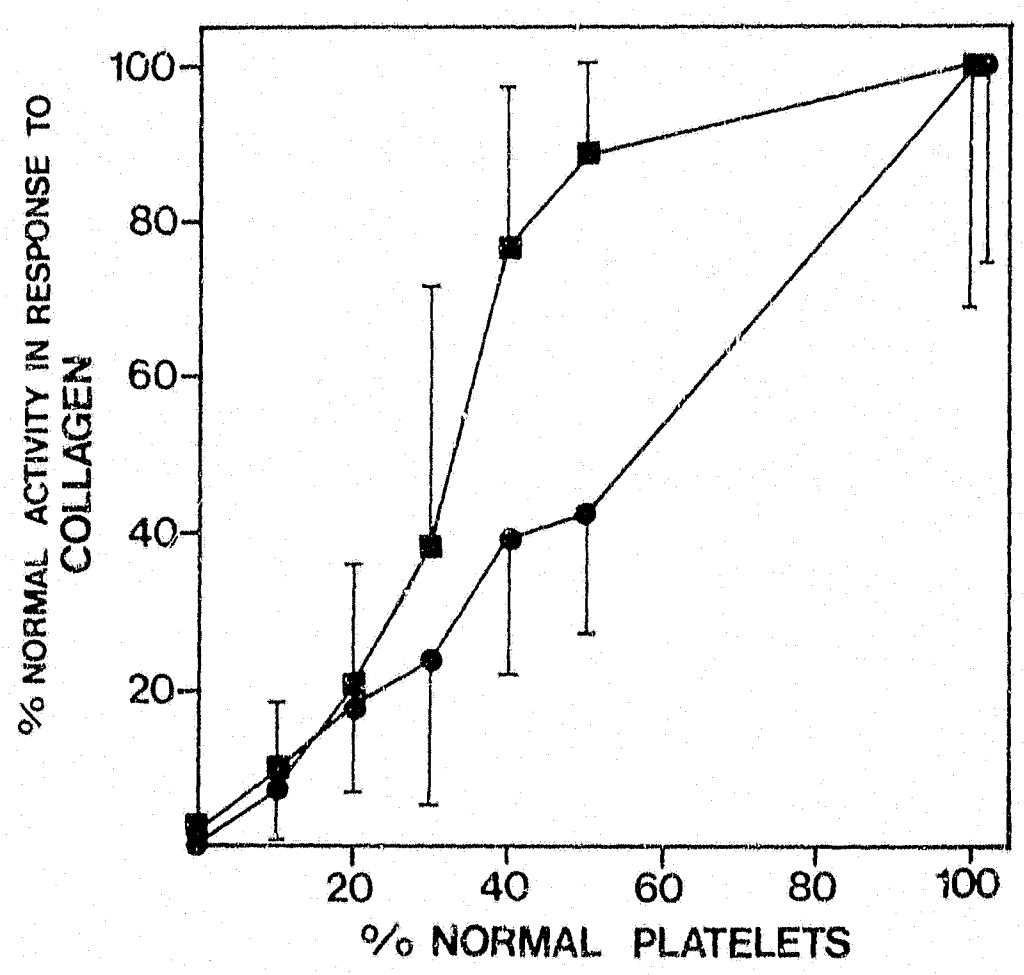


Fig. 4.15. Aggregation responses and TXB_2 production in mixtures of normal and aspirin inhibited platelets in response to collagen. Means ± S.D.derived from 13 normal subjects are shown. All results are calculated as the percentage of the aggregation response and of TXB_2 production in normal platelets.

■-Aggregation

●- thromboxane B_2

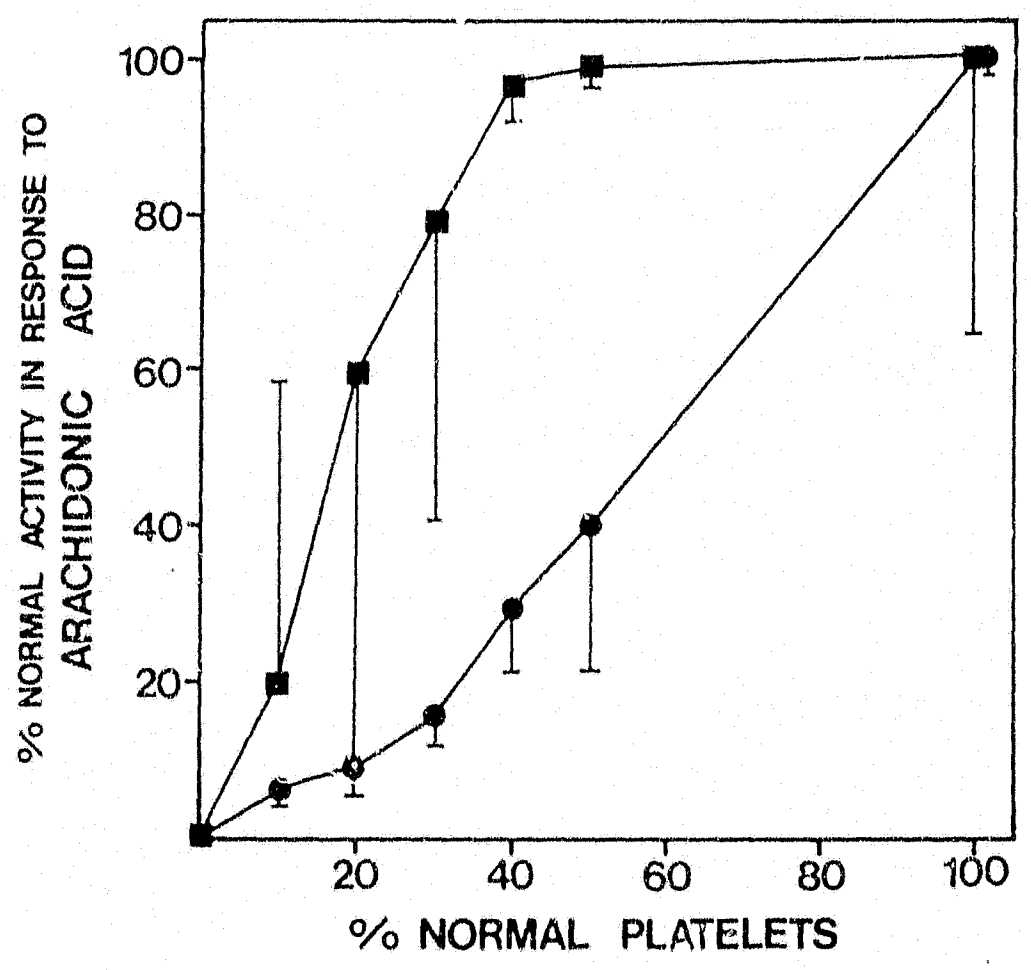


Fig. 4.16. Aggregation responses and TXB_2 production in mixtures of normal and aspirin inhibited platelets in response to arachidonic acid. Means ± S.D. derived from 13 normal subjects are shown. All results are calculated as the percentage of the aggregation response and TxB_2 production in normal platelets.

■ - Aggregation

● - TXB_2 production

4.3.3. Control experiment.

Aggregation responses of samples 1 (pre aspirin PRP), 2 (post aspirin PRP) and 3 (pre aspirin PRP plus post aspirin PPP) are shown in figures 4.17 - 4.20. When post aspirin PPP was added to pre-aspirin PRP, aggregation to all four agonists used in this study was not inhibited.

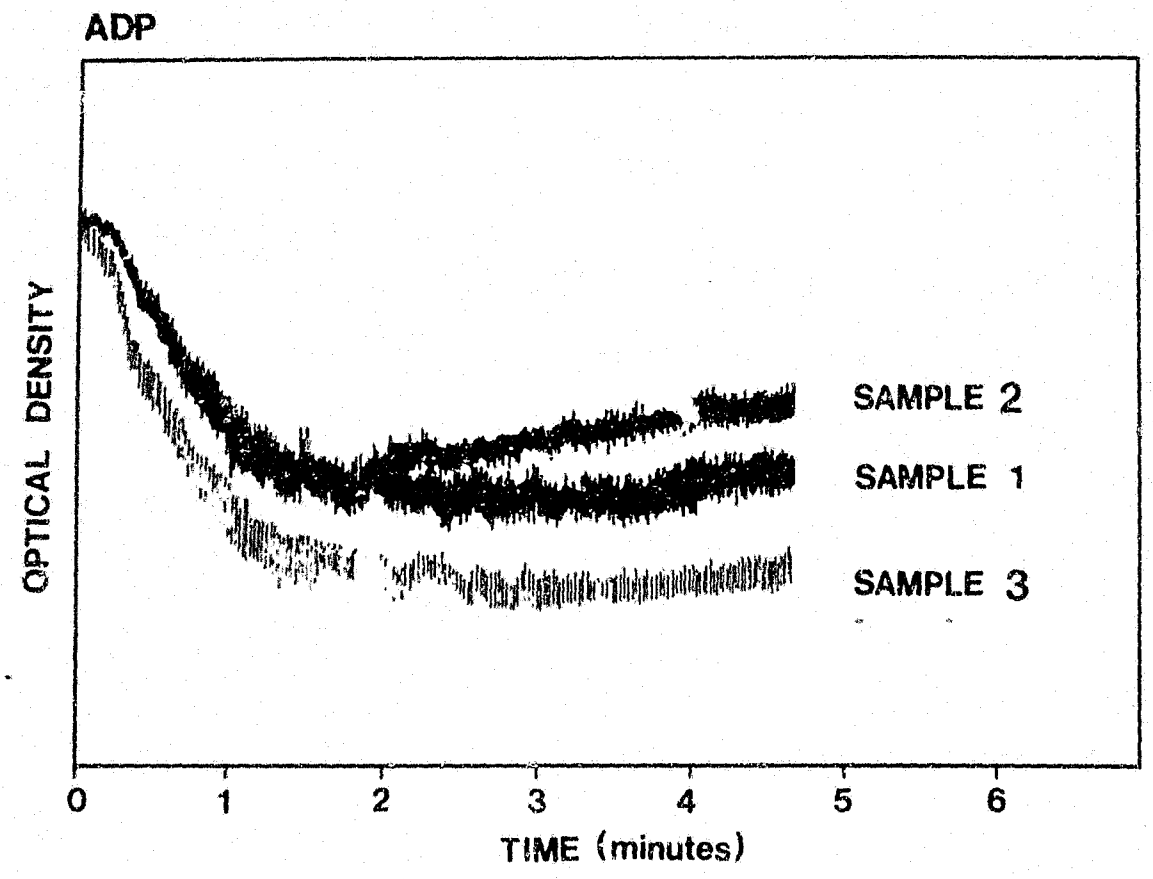


Fig. 4.17. A representative experiment showing the absence of circulating plasma aspirin. After 1 hour aspirinized PPP had no effect on normal (pre-aspirin) platelets when stimulated with ADP. (Sample = pre aspirin PRP; Sample 2 = post aspirin PRP showing only a primary wave of aggregation; Sample 3 = pre-aspirin PRP and post aspirin PPP).

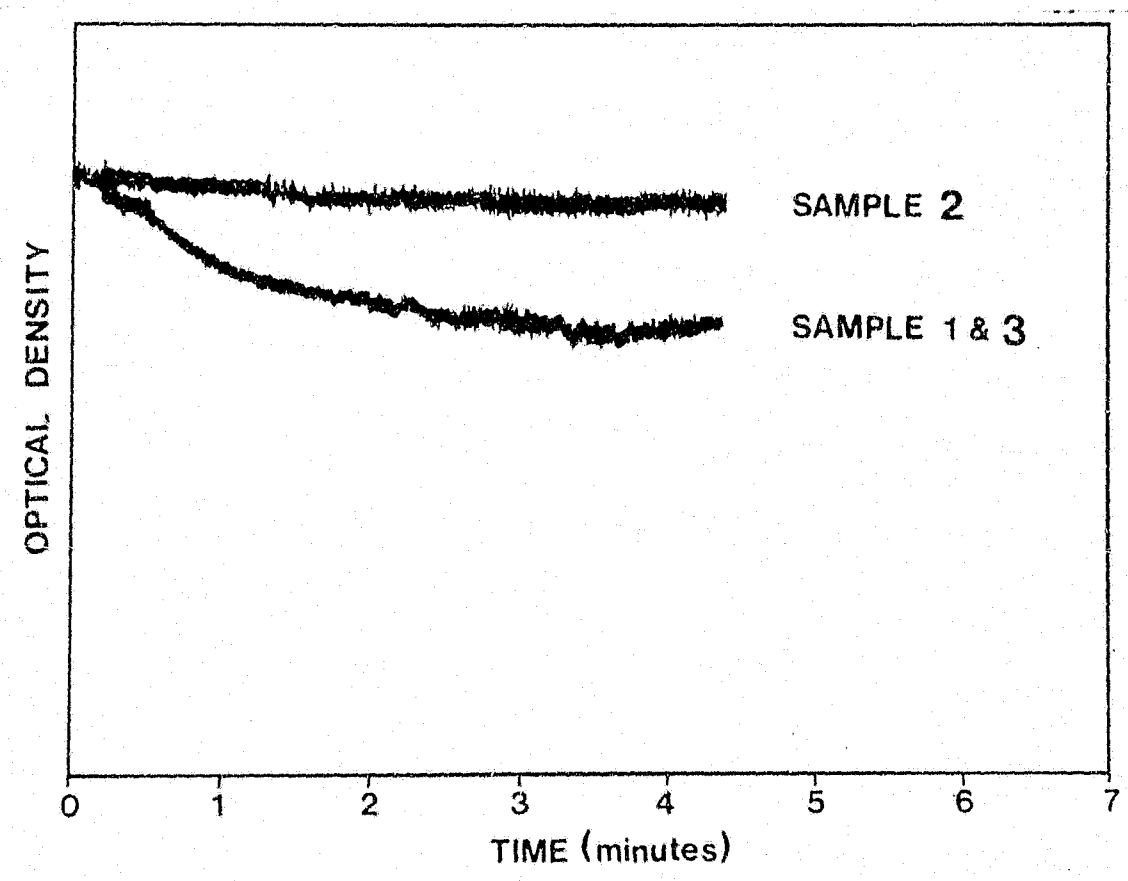


Fig 4.18. A representative experiment showing the absence of circulating plasma aspirin. After 1 hour aspirinized PPP has no effect on normal (pre-aspirin) platelets when stimulated with adrenalin. (Sample 1 = pre aspirin PRP; Sample 2 = post aspirin PRP; Sample 3 = pre aspirin PRP + post aspirin PPP).

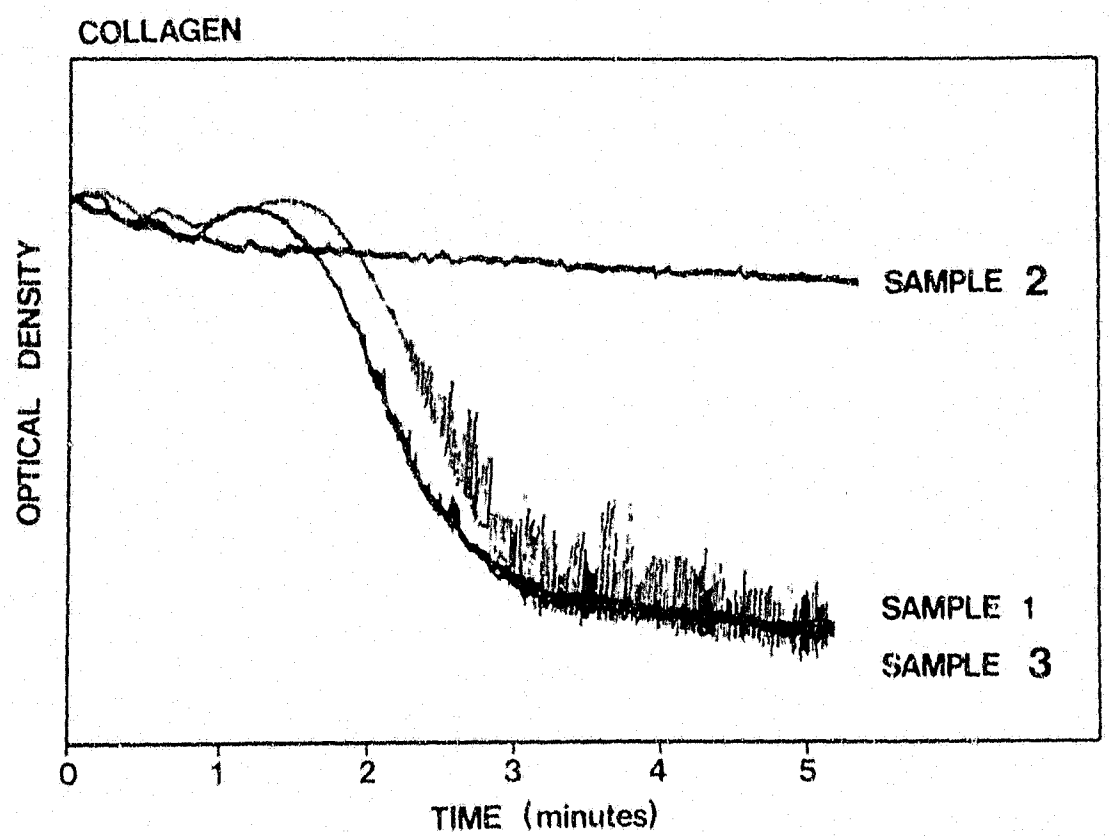


Fig 4.19. A representative experiment showing the absence of circulating plasma aspirin. After 1 hour aspirinized PPP has no effect on normal (pre-aspirin) platelets when stimulated with collagen. (Sample 1 = pre-aspirin PRP; Sample 2 = post aspirin PRP; Sample 3 = pre aspirin + post aspirin PPP).

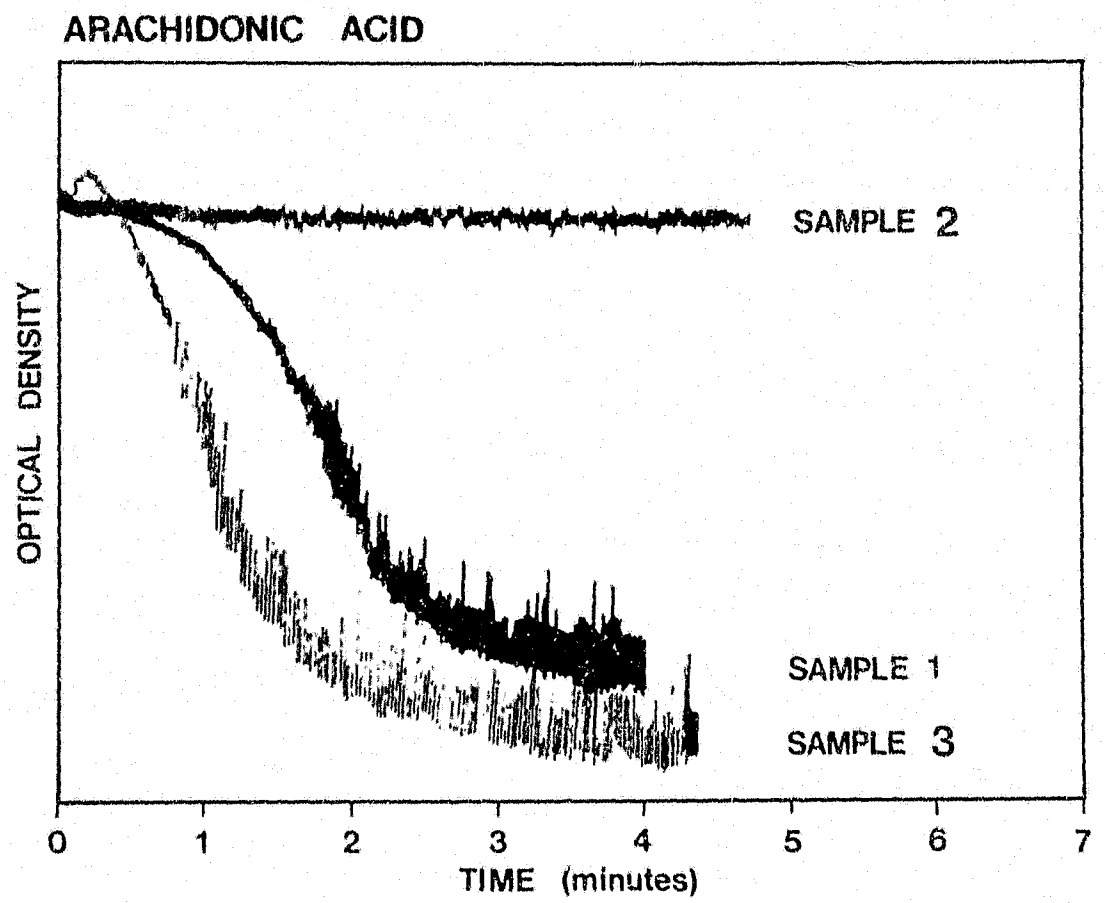


Fig 4.20. A representative experiment showing the absence of circulating plasma aspirin. After 1 hour aspirinized PPP has no effect on normal (pre-aspirin) platelets when stimulated with arachidonic acid (Sample 1 = pre-aspirin PRP; sample 2 = post aspirin PRP; sample 3 = pre aspirin PRP + post aspirin PPP).

4.3.4. Effects of 20mg aspirin

4.3.4.2. Platelet aggregation

Mean aggregation responses before and after aspirin ingestion, expressed as the percentage of the initial aggregation responses are shown in Table 4.4.

All subjects showed a fall in aggregation responses to all four agonists after 2 weeks. At the end of four weeks aggregation responses had improved slightly in the cases of adrenalin, collagen and arachidonic acid but there was no improvement in the response to ADP. The differences betwen the 2 and 4 weeks responses for the whole group were however not statistically significant.

In 2 of the subjects (Mr. Da and Mr.E) the responses were depressed after two weeks on 20 mg ASA daily but showed escape from the ASA effect after 4 weeks. These two subjects were given 40 mg of aspirin for the next 4 weeks. At the end of this second period their responses were suppressed to the same extent as they were after 2 weeks of either the 20 mg or the 40 mg dose. (Table 4.5)

4.3.4.2. Bleeding times

The mean bleeding time rose from 6.0 ± 1.0 to 8.5 ± 3.0 minutes after two weeks and was 10.0 ± 3.9 minutes after four weeks. Differences were significant ($p < 0.05$)

4.3.4.3. Thromboxane B_2

Thromboxane B_2 production after 20mg aspirin is shown in table 4.6.

Table 4.4. Aggregation responses after 20 mg aspirin/day. Results are expressed as a percentage of the initial aggregation response. Mean ± S.D.are shown

	ADP	COLL	EPI	AA
Week 2	62±40	30±16	43±19	10±3
Week 4	62+39	42±39	60±38	33±42

Table 4.5. Aggregation response to daily doses of 20mg and 40mg in two subjects expressed as a percentage of the initial response.

		2 Weeks				4 weeks			
Subject	Dose	AA	ADP	EPI	COLL	AA	ADP	EPI	COLL
Da	20mg	11	92	49	24	30	100	100	38
Da	40mg	2.5	78	84	37	9	81	42	89
E	20mg	6	18	23	21	100	64	52	100
E	40mg	4.4	30	23	20	17	45	30	33

Table 4.6. TXB_2 Production before and after 20 mg ASA daily (pmoles/ml plasma)

	ADP	Collagen	Epinephrine	AA
Before	4.7 ± 2.6	10.2 ± 2.8	8.9 ± 10.7	130.8 ± 62.4
2 weeks	1.1 ± 0.9	1.2 ± 0.7	0.6 ± 0.6	2.2 ± 1.8
4 weeks	2.0 ± 2.3	2.3 ± 2.5	1.1 ± 0.8	15.6 ± 18.9

Table 4.6. TXB_2 Production before and after 20 mg ASA daily (pmoles/ml plasma)

	ADP	Collagen	Epinephrine	AA
Before	4.7 ± 2.6	10.2 ± 2.8	8.9 ± 10.7	130.8 ± 62.4
2 weeks	1.1 ± 0.9	1.2 ± 0.7	0.6 ± 0.6	2.2 ± 1.8
4 weeks	2.0 ± 2.3	2.3 ± 2.5	1.1 ± 0.8	15.6 ± 18.9

4.4 DISCUSSION

Before discussing the significance of the results obtained in this study two technical aspects warrant consideration. The production of MDA after aspirin ingestion was reduced to an average of 34% of the basal load on the second day. Complete inhibition of MDA did not occur suggesting that either the cyclo-oxygenase was only partially inhibited or that the assay procedure was not specific. The latter explanation is probable since it has been shown that lipid peroxides can react with thiobarbituric acid (Pryor et al., 1976; Best et al., 1980), and it was found that a single dose of 325mg aspirin inactivated 90% of cyclo-oxygenase and that this effect persisted for 48 hours or more (Burch et al., 1978). The second aspect concerns the possibility that the post aspirin blood samples contained sufficient aspirin to influence aggregation in the mixtures of pre- and post aspirin PRP. When post aspirin PPP was added to pre-aspirin PRP aggregation to all four agonists used in this study were not inhibited. This experiment is in keeping with pharmaco-kinetic data showing that the half life of aspirin in plasma in vivo is 13 to 20 minutes (Kelton and Hirsh, 1981b). It is therefore very unlikely that the mixing experiments were affected by residual aspirin in the post-aspirin specimens.

The results of these studies indicate that an interaction

between normal and aspirin treated platelets can occur and that this may have a bearing on the frequency of aspirin dosage necessary to achieve a prolonged inhibition of platelet function.

Ex vivo studies indicated a return to normal aggregation responses several days before all aspirin treated platelets were likely to have disappeared. In four subjects the measured mean platelet survival times were 2-7 days longer than the times required for normal aggregation to return (Table 4.2). The suppression of aggregation responses in the ex vivo studies was incomplete in most of the subjects in that although the responses to collagen and AA were abolished within 24 hours those to ADP and adrenalin were reduced but not absent. At 72 hours there was very little evidence of recovery of function, by 96 hours partial recovery was evident and full recovery occurred within 5 to 8 days in all cases. At the time full recovery was present the proportion of new platelets in the circulation was likely to be 50% or more. Partial recovery occured sooner, at a time when the proportion of new platelets could have been as low as 35%. When the proportion of new platelets was less than this there was no evidence of functional recovery although the production of MDA had risen from its nadir 3 days after aspirin ingestion at which time the proportion of normal platelets could be 30% or less.

The *in vitro* studies showed that in many instances partial restoration of aggregation to adrenalin and ADP occurred with as little as 10% normal platelets in the mixture of normal and aspirin treated platelets. In all except one case at least 20% normal platelets was needed for partial restoration of aggregation to collagen and AA. Full restoration of function required at least 10% of normal platelets for ADP, 20% for adrenalin, 40% for collagen and 30% for AA.

Thromboxane B_2 production did not reach normal values even when the proportion of normal platelets was raised to 50% of the mixed platelets. The normal platelets did not appear to cause TXB_2 production in tho inhibited platelets. The increase in TXB_2 production as the proportion of normal platelets in the mixture was increased lagged behind the increase in aggregability of the mixed platelets. This would indicate that normal aggregation occurred with considerably less than maximal TXB_2 production.

These results contrast with those of Cerskus *et al.*, (1980) who found that 10% of normal platelets was sufficient to restore full aggregation after AA stimulation in a mixture of normal and aspirin platelets. They also reported partial restoration of serotonin release by addition of 10% normal platelets to aspirin

treated platelets. Their work was carried out with rabbit platelets, and therefore a species difference might account for the different results we obtained with human platelets.

The ex vivo experiments in this study demonstrate in most but not all cases a single dose of 300 mg aspirin will produce complete suppression of aggregation for 72 hours. In the in vitro experiments, however, there was evidence to show that in some cases 10% of normal platelets could restore some aspects of platelet function. Twenty-four hours after a dose of aspirin it is possible that 10% of normal platelets could have entered the circulation. This would imply that partial recovery of platelet function could occur as early as 24 hours after a dose. If consistent suppression of aggregation is to be achieved in all subjects this data would argue for a daily dose of aspirin. Support for this view can be deduced from the observation (Stuart et al., 1972) that platelets derived from donors who had ingested aspirin 36 hours before donations were able to correct the bleeding time of thrombocytopenic patients although platelets prepared 12 hours after aspirin ingestion were ineffective.

Considerable variation between individual subjects in the recovery of platelet function and in the proportion of normal platelets necessary to restore aggregation

responses was observed. Others (O'Brien, 1980; O'Brien and Jamieson, 1976; Hoogendijk and Ten Cate, 1980) have observed similar variation. Ideally therefore the effective duration of the aspirin effect should be determined for each patient. It is possible that the degree of variation may be even greater when disease processes are present. Furthermore the response to aspirin may change during the course of therapy. Periodic re-examination of the aspirin effect may then be necessary. In clinical practice this would be difficult and costly. A uniform dose would obviously be desirable.

If this view is accepted then this study would favour a dosage frequency of at least once a day. Longer intervals would permit recovery of platelet function in some cases.

Theoretical considerations suggest that an ideal dose of aspirin could inhibit platelet aggregation without affecting PGI_2 production by vascular endothelium (Marcus, 1977). The dose used in the present study may be too high since 150 mg of Aspirin can suppress PGI_2 production in venous segments (Preston et al., 1981b) although there was very little suppression of PGI_2 levels in venous blood after 3 minutes ischaemia of the forearm with a similar dose (Masotti et al., 1979). In another study (Hanley et al., 1981), a dose of 80 mg inhibited PGI_2 production in venous segments but 40 mg did not.

The effect of daily doses of 40 mg may however be cumulative and suppress both PGI_2 and TXA_2 production (Preston et al., 1982; Hanley et al., 1982). However when the suppression of TXB_2 was studied alone (Preston et al., 1982; Patrignani et al., 1982) daily doses of aspirin in the range of 20-40 mg may have seemed sufficient. However, Svensson and Samuelson (1983) reported that in some patients platelet aggregation was not inhibited by 50 mg aspirin daily although an extensive depression of TxB_2 was found. The response to AA was found to be an all or none response and only a very small amount of TxA_2 seemed to be needed to trigger the release reaction. Thus when monitoring threshold doses for low dose aspirin, platelet aggregation tests seem to be mandatory. The 20mg dose study showed that this dose is unlikely to inhibit platelet aggregation although TXA_2 production is inhibited.

In a study by Di Minno et al.,(1983), they showed the initiation of recovery of TxB_2 formation and platelet aggregation as early as 4 hours after the ingestion of 650 mg aspirin in response to a combined stimulus of AA plus ADP or epinephrine or collagen. Rao et al., (1980) have also studied some effects of pairs of aggregating agents. Using epinephrine in combination with ADP, thrombin, or AA, they observed irreversible aggregation in PRP from subjects who had ingested aspirin 10-16 hours previously.

If *in vivo* aggregation is likely to occur in response to a combination of agents it seems likely that the need for daily administration of aspirin would be even greater than our data suggest in order to achieve constant suppression of platelet function.

Despite the theoretical objection to large doses of aspirin, clinical benefits have been documented with daily doses of 1g or more (Verstraete, 1980; Salzman, 1982). It may be that aspirin has effects on platelets other than its inhibition of cylo-oxygenase (Buchanan *et al.*, 1982). Doses of 1g or more can also reduce the levels of Factor II, VII, IX, and X (Kelton and Hirsh, 1981a) and may therefore act as anticoagulants. The concept of a low dose of aspirin that would discriminate between the inhibitory effects on platelets and on endothelium, although attractive, may well be illusory. Even if such a dose exists, its clinical value has yet to be demonstrated.

4.5. SUMMARY

The effect of 300mg aspirin on platelet aggregation and MDA production in 7 volunteers indicated that partial recovery of platelet function occurred when approximately one third of the circulating platelets had been replaced by new (uninhibited) platelets. *In vitro* studies on mixtures of normal and aspirin inhibited platelets in 22 volunteers, indicated partial restoration of platelet aggregation and thromboxane B_2 production with as little as 10% of normal platelets in some subjects. Restoration of full function required a higher proportion of normal platelets. There were considerable variations between subjects. These data suggest that complete suppression of platelet functions in all normal subjects requires daily administration of the drug. Furthermore, a daily dose of 20mg aspirin was found to be insufficient to maintain adequate inhibition of platelet function in normal subjects.

CHAPTER 5

THE EFFECT OF LOW DOSE ASPIRIN ON PLATELET AGGREGATION IN FAMILIAL HYPERCHOLESTEROLAEMIA

5.1 Introduction

Familial hypercholesterolaemia (FH) occurs in it's heterozygous form with a prevalence of 1/60 in the S.A. white population (Seftel, et al., 1980) and is a major factor predisposing to premature atherosclerosis and myocardial infarction in adolescents and young adults. By the age of 50 years 51% of men with FH have suffered myocardial infarction, and by the age of 60 the figure has risen to 85% for men and is 58% for women (Slack, 1969). The rate of coronary artery disease is 25 times greater in FH patients than in their unaffected relatives (Jensen, 1967). Since the disorder of cholesterol metabolism is inherited, advanced atherosclerosis may be present at the time of clinical presentation, and the results of therapy designed to normalize circulating cholesterol levels are often unsatisfactory.

The most common cause of obstructive arterial disease is slowly progressive arteriosclerosis obstructing the blood flow to the lower limbs, often complicated by thrombosis (Jurgens et al., 1960).

Blood platelets may make an important contribution to the developrent of atherosclerosis. There are at least three mechanisms by which platelets may participate in atherogenesis (see 1.1.6.).

In recent years attention has been focussed on the role of platelets in the pathogenesis of occlusive coronary artery disease (Salzman, 1982). Patients with coronary artery disease show increased aggregation and activation of platelets in the coronary vascular bed (Mehta and Mehta, 1980). Activated platelets release TXB_2, which causes further aggregation and may result in arterial spasm (Burch and Majerus, 1979). Atherosclerotic plaques, the sites of platelet aggregation, may have a reduced capacity for producing prostacyclin (Sinzinger et al., 1979), which is a vasodilator and inhibits aggregation (Kulkarni et al.,1976; Moncada et al., 1977), thus permitting the unopposed action of platelet thromboxane A_2 and facilitating aggregation.

Abnormal platelet function and increased plasma thromboxane B_2 were reported in patients with ischaemic heart disease (De Gaetano, 1981; Dreyfuss and Zahavi, 1973), cerebrovascular disease (De Gaetano, 1981), diabetes mellitus (Dollery et al., 1979) and Hyperlipidaemia (Carvalho et al., 1974; Shattil et al., 1975; Shattil et al., 1977; Joist et al., 1979;

Bradlow et al., 1982; Chetty, 1983; Avi Viener et al., 1984; Zahavi and Zahavi 1985).

One approach to the prevention of the thrombo-embolic complications of atherosclerosis is the use of agents which depress platelet function, particularly platelet aggregation and Thromboxane A_2 production. Since the platelets of FH patients have been shown to be hyperaggregable (Carvalho et al., 1974; Shattil et al., 1975; Shattil et al., 1977; Joist et al., 1979; Bradlow et al., 1982; Chetty 1983; Avi Viener et al., 1984; Zahavi and Zahavi 1985) and because theoretical considerations suggest that a low rather than a high dose may be more effective in preventing thrombosis (Marcus, 1977), the effect of a low dose aspirin on platelet activity in FH patients and in normal subjects were compared.

5.2 Materials and Methods

5.2.1 Subjects

Two groups of subjects were studied. Group 1 consisted of 9 young untreated patients, 3 males and 6 females, with the heterozygous form of FH. The clinical and biochemical data of the patients are shown in table 5.1. Their mean age was 27 years (range 19 - 34 years). All had a family history of hypercholesterolaemia and 5 had thickened Achilles tendons. Two of them had mild exertional chest pain. Their mean serum cholesterol and low density lipoprotein (LDL) cholesterol values were 9.19 mmol/L (range 7,1 - 11,9 mmol/L) and 7.42 mmol/L (range 5.93 - 10.3 mmol/L) respectively. All had normal fasting glucose and creatinine levels, and all except 1 had normal resting electrocardiographic tracings.

Group 2 consisted of 9 healthy control subjects, 3 males and 6 females, with a mean age of 26 years (range 19 - 34 years). All had normal serum lipid values except for 1 whose cholesterol level was mildly raised. However, the results of his tests were similar to those of the other control subjects.

Table 5.1
Clinical and bio-chemical data of F.H. Type IIa Patients when first diagnosed

	Age	Sex	Clinical Features	Serum Lipid Studies (mmoles /litre) TC	LDL	HDL	TRG
1	28	F	Heterozygous. Angina grade 1 Dyspnoea on exertion ? atypical claudication	11,90	10,30	1,29	1,06
2	26	F	Heterozygous. Achilles Tendon	9,10	7,80	0,78	1,13
3	28	F	Heterozygous. Angina grade 1. Tendinous Xanthoma. Achilles Tendon and Knuckles	10,10	8,79	0,61	1,52
4	19	F	Heterozygous ESM grade 2/6	10,10	8,90	1,01	0,54
5	28	M	Heterozygous. Arcus senilis Tendinous and tuberous Achilles tendon	11,05	8,80	1,61	1,40
6	25	F	Heterozygous. Xanthelasma slight (R) eye. Achilles tendon. Ankle swelling	9,10	6,19	1,19	1,40
7	34	M	Heterozygous. Angina grade 1 Arcus eyes. Tendininous Xanthoma	7,10	3,93	0,73	0,97
8	23	F	Heterozygous No symptoms	6,89	5,93		0,90
9	28	M	Heterozygous Angina grade 1	7,41	6,19	0,81	0,90

Angina grade 1 :- pain provoked by walking up hills or flight of stairs

Author Chetty N
Name of thesis Drug and dietary modification of platelet function 1985

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