THE EFFECT OF SODIUM TETRADECYL SULPHATE ON COAGULATION AND ENDOTHELIUM

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TO MY PARENTS

HILDA AND MARX JACOBSON

DECLARATION

This is to certify that this thesis, presented for the degree of Doctor of Philosophy, is my own work and has not been submitted for a degree at any other university.

BARRY FRANK JACOBSON

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ABSTRACT

Despite having been initially described more than fifty years ago, sclerotherapy of oesophageal varices has only relatively recently become regarded as one of the primary modalities both to control bleeding oesophageal varices and to prevent recurrent bleeding. Sclerotherapy, however, is associated with numerous complications and its exact mechanism of action, particularly that pertaining to its effect on haemostasis, has to date been poorly documented. One of the problems of comparing the various trials has been the diversity of both the technique and the type and concentration of the sclerosants used.

This thesis investigated the effects of Sodium Tetradecyl Sulphate (S.T.D.), a sclerosant, on haemostasis. Haemostatic function was determined in relation to the effect of varying concentrations of S.T.D. on platelet function, the coagulation pathway and endothelium.

Platelets, which play an integral role in the initiation of thrombus formation, were shown to be completely lysed by concentrations of S.T.D. likely to be found within varices injected with standard dosage S.T.D. However, on dilution, a potent and altogether unexpected activation of platelets was documented by platelet aggregometry. The aggregation pattern comprised both primary and secondary waves and was similar to that seen following platelet aggregation induced by physiological agonists, such as adrenaline and ADP.

In further in vitro studies, involving time sequence phase contrast light microscopy, designed to simulate the gradient of dilution occurring in varices as the distance from the injection site increases, a similar pattern of local high dose lysis, with distal low dose aggregation was exhibited with S.T.D.

S.T.D. was shown to destroy functional coagulation parameters of the intrinsic and extrinsic pathways at dosages which are used clinically. At dilute dosages these parameters were not altered. There was, however, a selective reduction in Protein C, which is a physiological anticoagulant. This suggested that a hypercoagulable state occurs, not at the site of injection but rather further downstream. As hypercoagulable states are notoriously difficult to document, thromboelastography was performed to try and demonstrate the presence of hypercoagulability. Thromboelastography did provide further evidence that at low dosages hypercoagulation occurred which was distinct from the marked hypocoagulation which resulted from addition of relatively high dosages of S.T.D.

An umbilical vein model was developed to investigate the effect of S.T.D. on the endothelium. This model showed extensive denudement of endothelium with exposure of subendothelium which still contained von Willebrand factor. The extent of the damage induced by standard dosage S.T.D. was extensive, but could be limited and

controlled by diluting S.T.D. tenfold.

All the above evidence suggested that the dilute S.T.D. would be not only as effective as undilute sclerosant at causing endothelial damage at the site of injection, but it would also be more thrombogenic as it would activate platelets and cause a hypercoagulable state. In a pilot study, tenfold diluted S.T.D. was shown to be as effective as 3% S.T.D. in controlling varices which are acutely bleeding. There was a lower complication rate and to date there have been no failures with this sclerosant.

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CHAPTER 1

1. INTRODUCTION

1.1 PORTAL HYPERTENSION

1.1.1 History

The Egyptians were the first to document an association between liver disease and ascites in the Papyrus Ebers (Bryan, 1931). The first report in the literature of a fatal variceal bleed was in 1840 (Power). Although he documented rupture of varices of the oesophagus, he failed to connect the pathophysiology to liver disease. Sixty years later, an autopsy series of Preble (1900) showed the inter-relationship between variceal bleeding and liver disease. In 1906, two French workers were the first to use the term Portal Hypertension, after realising that cirrhosis of the liver was connected to an increased portal venous pressure (Gilbert, 1906).

1.1.2 Aetiology

The most important aetiological causes of portal hypertension in Southern Africa include the following categories:

1.1.2.1 Prehepatic - Presinusoidal

Portal vein thrombosis which is usually a result of sepsis in the neonatal period is an important cause of portal hypertension especially in children (Sherlock, 1989).

1.1.2.2 Intrahepatic - Presinusoidal

Schistosomiasis is worldwide one of the most common causes of portal hypertension (Henderson et al, 1986). In Southern Africa, particularly along the eastern coast, it is responsible for a large number of cases of portal hypertension in rural communities (Donnely et al, 1984). The parasite causes fibrosis of the terminal portal venous radicles (so called pipe stem fibrosis) but leaves hepatic function and architecture essentially intact (Henderson et al, 1986). Liver function in these patients is usually completely normal. Other causes of intrahepatic presinusoidal block are congenital hepatic fibrosis and early primary biliary cirrhosis (Sherlock, 1989).

1.1.2.3 Intrahepatic - Sinusoidal

In Southern Africa, the most common cause of portal hypertension amongst the White population group is cirrhosis (Kew et al, 1984). Cirrhosis is derived from the Greek word "kirrhos", which means orange-yellow discolouration. Liver cirrhosis is defined by the World Health Organization as a diffuse process characterised by fibrosis and the conversion of normal architecture into structurally abnormal nodules.

Anthony et al (1977), who were responsible for the definition, felt that cirrhosis is a chronic progressive condition and that regression or reversability was a rare event and subject to doubt. Recent experimental data however suggests that macronodular cirrhosis might well be reversible (Ishii et al, 1990).

The current histological criteria for making a diagnosis of cirrhosis of the liver are hepatic parenchymal necrosis, fibrosis due to active formation of connective tissue and nodular regeneration of liver cells (Anthony et al, 1977).

The most frequent cause of liver cirrhosis amongst the White population of Southern Africa is alcohol (Kew et al, 1984). Alcohol related liver cirrhosis is becoming more prevalent in the urban Black population (Isaacson, 1978; Asvat et al, 1981). Another important cause of liver cirrhosis in Blacks, particularly in rural areas, is macronodular cirrhosis probably post viral in nature (Asvat et al, 1981).

Iron overload is also an important cause and is often a contributory factor in many of these patients (Isaacson, 1978).

Alcohol abuse is becoming an increasing problem in Southern Africa (de Miranda, 1988). In 1982 the Human Science Research Council survey on South African drinking problems showed the following findings on alcohol consumption:

White Population	Male 91%	Female 76%
Coloured Population	Male 60%	Female 28%
Black Population	Male 54%	Female 22%
Indian Population	Male 45%	Female 4%

In the Republic of South Africa liquor sales for the year ending March, 1987, were:

Spirits (including imported)	83 million litres
Wine (including fortified)	310 million litres
Beer	1500 million litres

The Institute for Race Relations estimates that at that time the total population of South Africa was approximately 35 million people. This then equates to an annual consumption of 2.4 litres of spirits, 8.9 litres of wine and 42.9 litres of beer per capita. These figures include children.

Worldwide it has been found that a constant percentage of people who use alcohol will become dependent. This figure is 6 to 8% and would appear to be constant despite varied environmental conditions (de Miranda, 1988). So as the race groups, other than Whites who would appear to have reached a saturation point, increase their alcohol intake, the number of alcohol dependent diseases will continue to increase. Therefore the number of patients with alcohol induced cirrhosis will also increase.

The relatively small study which was performed at Baragwanath Hospital and presented later in this thesis confirmed that alcohol was the most common cause of bleeding oesophageal varices in patients who presented at that hospital.

1.1.3 Portal Hypertension - Definition

Portal hypertension is defined by Hobbs as a portal blood pressure of greater than 15 cm H_2O and is resultant upon an increase of either the volume of portal blood flowing to the liver or an increased resistance to the portal flow (Hobbs 1986). The normal portal vein pressure is defined at 5-10 mm Hg (Henderson and Warren, 1986). Therefore any elevation of the above would fulfil the criteria of a diagnosis of portal hypertension.

The pressure may be measured directly by means of transhepatic portography, umbilical vein catheterisation or spleno- portography. The pressure may also be measured indirectly by retrograde catheterisation of the hepatic vein and by measuring wedged and free hepatic venous pressure.

The indirect method (i.e. the wedged minus the free pressures) would appear to approximate the direct pressure accurately enough for it to be used routinely as it is less invasive than the direct method. Direct portal venous measurements in patients with portal hypertension are usually elevated in the region of 15-40 mm Hg and indirect measurements are 10-30 mm Hg.

Hepatic vein wedge pressure is not recommended for assessing presinusoidal portal hypertension as it will be normal in such cases. In patients without disease, hepatic vein wedge pressure is marginally lower than portal pressure. Patients who have intrahepatic shunting due to alcoholic cirrhosis have hepatic wedge pressures which approximate portal pressures (Hoefs et al, 1990). However, Valla et al showed in 1984 that after administration of propranolol, hepatic wedge pressure was not an accurate indicator and that direct portal venous pressure measurement was the technique of choice in

assessing the response to the administration of agents which alter splanchnic circulation.

1.1.4 Portal Hypertension - Pathophysiology (Mahl and Groszmann, 1990)

Portal hypertension is due to an increase in portal pressure.

Pressure is defined by Ohm's law as

$$P = k \times Q \times R$$

where P is the change in pressure, Q is the flow, R is the resistance, and k is the constant.

Poiseuille's law states that

$$R = k8nL/ r^4$$

where R is resistance, k is the constant, n is the coefficient of velocity, L is the length of the vessel and r is the radius. From Poiseuille's law it is obvious that r (the radius) plays a most important role in the human body. Resistance is one of the only variables in the biological system, as all the other factors remain relatively constant.

The liver is the main source of resistance to portal blood flow, and is unable to control this flow. The actual site of the resistance in the liver is thought to be either the small portal veins, hepatic sinusoids or the terminal hepatic venules. The current theory for the cause of the raised pressure is thought to be due to deposition of fibrous tissue around the

terminal hepatic venule and adjacent sinusoids (Mahl et al, 1990). A separate theory is that the hepatocyte enlarges when damaged (Blendis et al, 1982). This latter theory might explain why, in some cases, precirrhotic portal hypertension, consequent upon alcoholic hepatitis improves with abstinence.

All the above theories suggest that portal hypertension is due to an increased resistance. However, with the development of portal hypertension, portasystemic shunting occurs and may divert most of the blood from the portal system to the systemic system (Vorobioff et al, 1983). Despite the shunting, portal hypertension persists. Vorobioff et al (1983) found that there is a relative increase in splanchnic blood flow and while it does not initiate portal hypertension, it plays a role in maintaining it.

These findings are in keeping with those of Banti (1883) who described a group of patients with splenomegaly, anaemia and leukopenia and postulated that the pathophysiology of their portal hypertension was related to increased splenic arterial blood flow, i.e. he described an increased forward flow was causing the portal hypertension (Mahl et al, 1990).

Cohn et al (1972) felt that the collaterals were the cause of the hyperdynamic circulation. Sikuler (1986) showed that the degree of shunting did not correlate well with the flow. The precise cause of the hyperdynamic circulation has not been fully elucidated although glucagon, bile acids and an expanded plasma volume have all been incriminated (Mahl et al, 1990). Increased splanchnic flow has recently been attributed to elevated levels of vasodilators in the circulation as well as diminished sensitivity of the splanchnic vasculature to endogenous vasoconstrictors (Eckhauser et al, 1991).

Sivak et al (1990) maintain that the prevalence of oesophageal varices in patients with cirrhosis is difficult to determine. However, it has been shown that approximately 60% of patients with portal hypertension have varices if one uses autopsy series (Olson, 1972). Using endoscopic techniques, Conn et al (1968) documented varices in 40-50% of patients with cirrhosis. Lebrec et al (1980), in a series of 100 patients with histologically documented alcoholic liver cirrhosis, demonstrated varices in 84 patients using a combination of x-ray examination and endoscopic assessment.

The portasystemic shunting occurs at many sites (McIndoe, 1928). These include the coronary and short gastric veins, the azygous veins, the intercostal veins, the paraumbilical plexus, the retroperitoneal veins, the haemorrhoiadal veins and, most importantly, from a clinical point of view, the oesophageal veins.

1.1.3 Anatomy of the Portal Venous System (Meyers, 1986)

The superior mesenteric vein, which drains the intestinal bed, is joined by the splenic vein behind the neck of the pancreas to form the portal vein. The inferior mesenteric vein drains into the splenic vein a few centimetres proximal to where the splenic vein enters the superior mesenteric, although this is variable. Another vein of importance is the left gastric vein (coronary) which also enters in the region of the confluence although once again its position is variable.

The portal vein then runs for approximately 6 to 8 cm and lies posterior to both the common bile duct as well as the hepatic artery. It lies in the free edge of the lesser omentum. On arriving at the liver it branches into left and right branches. The left branch supplies the left lobe, caudate and quadrate lobes. The right branch supplies only the right lobe.

The venous blood from the portal system mixes with arterial blood from the hepatic arterial branches only at sinusoidal level. The portal venous blood is responsible for two-thirds of the oxygen requirement of the hepatocyte.

1.1.6 The Anatomy of the Veins of the Oesophagus

The first comprehensive study of the venous anatomy of the oesophagus was published in 1951 by H. Butler. He studied 21 foetuses, 17 cadavers and 2 fresh specimens. He used India ink injections on the foetuses and Neopreone on the fresh specimens. His subsequent classification has stood the test of time. The most recent study by Kitano et al (1986), has elaborated on Butler's original work and the findings are of clinical relevance, particularly to sclerotherapy and its effect on the oesophageal varices. Kitano studied ten fresh human cadavers, five of whom had known portal hypertension with oesophageal varices due to alcoholic cirrhosis. Because no reference is made to therapy in the last group of patients, one assumes that these patients did not undergo sclerotherapy.

The normal oesophagus has 4 distinct layers of vein (Kitano et al, 1986):

Intra-epithelial channels

Superficial venous plexus

Deep intrinsic veins

Adventitial veins

The intra-epithelial channels are made up of fine vessels which lie inside the epithelium and are actually the veins which drain the capillary network. These veins drain at right angles to the superficial venous plexus. This plexus has free communication with its gastric equivalent. The following vein is the deep intrinsic vein. There are usually three to five of these and they are often inter-connected to each other, as well as to the superficial venous plexus. They also form direct connections with their gastric counterpart.

The adventitial veins are connected by perforating veins to the deep intrinsic plexus. The perforating veins are found most often at the area adjacent to the oesophago-gastric junctions. The adventitial veins are numerous and are found in the peri-oesophageal region.

1.1.6.1 <u>The Anatomy of Oesophageal Varices</u>

(Kitano et al, 1986)

In patients with portal hypertension all of the above veins are significantly dilated.

The most marked would appear to be the three to five deep intrinsic venous channels. They do not appear to have marked communication with each other and communicate less frequently with the superficial venous plexus.

The superficial venous plexus is also dilated and has marked communication with the intra-epithelial channels.

The adventitial veins are also dilated and communicate with the deep vein by dilated perforating veins. This arrangement of the veins at the oesophago-gastric junction would appear to be more longitudinal in these patients. The deep intrinsic veins sometimes are so large that they displace the superficial venous plexus and lie just below the epithelium. The appearance of a dilated superficial venous plexus immediately on top of a dilated deep intrinsic vein results in the clinical appearance of the so called varices upon varices.

1.2 THE CAUSE OF BLEEDING VARICES

The presence of varices does not necessarily result in haematemesis. Several factors are thought to play a role (Mahl et al, 1990). These are overlying mucosal damage; size, degree and protrusion of the varices; portal pressure; and finally actual variceal pressure.

When one considers these independently, the mucosal damage theory has been widely propounded. The erosive theory was first suggested by Waagensteen (1945) who showed in an experimental animal model that erosion and ulceration occurred more frequently in response to histamine when the splenic or portal vein was occluded. However by 1961 Liebowitz had reviewed all the available literature at that stage and felt that the mucosal damage was overestimated and played a minimal role in causing rupture of varices. He also suggested that the oesophagitis and ulceration were a result of venous congestion rather than the effect of acid on the mucosa. Orloff et al (1963) biopsied oesophageal mucosa overlying varices at the time of transthoracic variceal ligation for bleeding varices and found histological evidence of mucosal damage in only 1 out of 20 patients on whom he operated. Eckhardt et al (1979) showed that the incidence of oesophageal reflux is not increased in patients, which would support the notion that oesophagitis seems to play little, if any role, in precipitating the haemorrhage. The above is confirmed in the study by MacDougall et al (1982), who showed in a double blind study that cimetidine was no different from placebo in

prevention of variceal haemorrhage. This was despite a relatively high dosage of cimetidine, viz. 1.2 grams daily. The authors also showed no difference in oesophageal function in patients with varices (both cirrhotic and noncirrhotic) and healthy control subjects.

Sherlock (1990) feels that reflux oesophagitis has little if any role in initiating rupture of varices. The above obviously does not hold for patients who have had sclerotherapy. Some of these patients have marked problems with oesophageal motility, which is discussed later, and reflux oesophagitis might certainly play a role in bleeding which occurs in these patients.

The second factor which could play a role is the size of the varices and the degree of the protrusion. According to Laplace's law

$$T = kPr$$

(T = Tension , k = constant, P = Pressure, r = radius), one would expect that the varix which has a large radius would be much more likely to rupture than a similar varix with a smaller diameter. This hypothesis has been borne out by Palmer and Brick (1956) who showed that out of 18 patients who were actively bleeding, 78% had varices greater than 6 mm. Baker (1959) performed a longitudinal study and his findings corroborated those of Palmer in that the incidence of bleeding was doubled in patients with large diameter varices.

Large varices also protrude into the lumen of the oesophagus. This results in less support from surrounding tissue. Polio et al, (1986) have shown that the surrounding tissue itself is an important factor in that when a vessel is surrounded by tissue, its radius is smaller at a given pressure and therefore less likely to rupture.

The next factor to consider is portal pressure. The varices are a direct result of an increase in portal pressure. One would therefore expect that the risk of bleeding from these varices would be proportional to the pressure. However, the correlation between the absolute amount of portal hypertension present and the risk of bleeding is controversial in the literature.

Adamsons et al (1977) showed that recurrent or prolonged bleeding in patients with higher pressures occurred more often than in a similar group of non-bleeding patients with portal hypertension who had a lower pressure. Burcharth (1980) showed that the portal pressure, measured transhepatically, was higher in patients who had bled within the previous 90 days. However, there have been numerous studies which have failed to show this correlation, e.g. Smith-Laing et al (1980) investigated 64 patients with variceal haemorrhage who had portal pressures from 11 to 45 mm Hg (mean 30.2) and found no correlation between portal pressure and the number or size of bleeding episodes.

There was no correlation between the portal pressure and the size of the gastro-oesophageal collaterals. They also found no relationship between portal pressure and total collateral circulation. Based on this study, and despite the previous discrepancies in the literature, it would appear that the degree of portal hypertension is a poor predictor of size or amount of variceal haemorrhage. In a more recent study, Vinel et al (1986) have shown that long term survivors had lower portal pressures than patients who died within 2 weeks of their haemorrhage. A large well performed study by Garcia-Tsao et al (1985) revealed that a pressure of greater than 12mm Hg is usually present before bleeding occurs. They examined 93 patients with liver cirrhosis and found that the hepatic vein pressure gradient was less than 12mm Hg in the 49 patients who did not bleed.

Endoscopic measurement of actual variceal pressure has also been evaluated and pressures greater than 12 mm Hg have been associated with a higher incidence of bleeding (Hoefs et al, 1990).

Endoscopic evaluation of the varices has been used as a predictor of impending haemorrhage (Beppu et al, 1981). The Japanese Research Study for portal hypertension has developed a classification for recording endoscopic findings (see Figure 1.1). Sivak et al (1990) feel that the mere presence of any red marking is associated with a risk of bleeding.

Azygous vein blood flow has been measured in patients with portal hypertension. The reason is that blood flowing from the portal system in portal hypertension drains via collaterals into the azygous system and correlates with gastro-oesophageal blood flow (Hoefs, 1990).

Bosch and Groszman in 1984 described the technique using a thermal dilution principle. They investigated 10 patients with alcoholic cirrhosis, and showed that the flow in patients was significantly higher in those patients who had not undergone decompressive surgery and that a high flow rate was also associated with bleeding.

1. Red Colour Signs
   (a) Red wale markings (Grade 1-3)
   (b) Cherry red spots (Grade 1-3)
   (c) Haematocystic spots
   (d) Diffuse redness

2. Fundamental Colour
   (a) White
   (b) Blue

3. Form
   (a) Small straight varices
   (b) Enlarged, tortuous, occupy less than one-third of the lumen
   (c) Largest, coil-shaped, occupy more than one-third of the lumen

4. Location
   (a) Lower third
   (b) Middle third below tracheal bifurcation
   (c) Upper third above tracheal bifurcation.

5. Adjunctive Finding
   (a) Erosion present

FIGURE 1.1

General Rules for Recording Endoscopic Findings on Oesophageal Varices according to the Japanese Research Society for Portal Hypertension

1.3 THE HISTORY OF SCLEROTHERAPY

The first description of sclerotherapy of oesophageal varices was in 1939 by Clarence Crafoord and Paul Frenckner. They described a case of a 16 year old girl who presented at the Sabbatsbergs Hospital with "great vomitings of blood". The history of the patient was non contributory. Clinical examination revealed a large liver at that stage. The patient was treated with "coagulen" and a blood transfusion. The patient had aniso-poikilocytosis on admission. After 2 months the patient developed a palpable spleen and Crafoord performed a splenectomy in June, 1934. At the time of laparotomy the findings were "a number of enlarged soft glands along the upper edge of the pancreas but otherwise the abdomen disclosed no pathological changes. The course was free from complications." However 2 years later the patient again presented with haematemesis. X-rays were done, as well as oesophagoscopy which was performed by Frenckner. He described "a collection of grape-like varices located mainly in the posterior and left parts of the oesophagus, wended their way downwards in two cord-like formations, each as thick as the little finger ...". Crafoord thought it would be of benefit "to produce collapse of the veins and varices through injections of quinine according to the same principles and doses approved at the surgical department for haemorrhoids". Frenckner designed a special needle to deliver the sclerosant via the oesophagoscope. It was decided to begin proximally, and if the procedure was effective, to continue distally.

Two millilitres of quinine-urotan was injected into each of 3 varices. They reported that at repeat oesophagoscopy the varices were "in a collapsed condition, forming an undulated, firmer part of the oesophageal wall". The procedure was repeated on numerous occasions and at the time of going to press, i.e. 3 years later, the patient had not had a relapse.

The first report of sclerotherapy of oesophageal varices from the United States was from Herman J. Moersch from the Mayo Clinic in 1941. In his paper he notes that in 1929 Rowntree, Walters and McIndoe advocated, at [illegible] hospital, ligation of the coronary vein as a possible method of preventing bleeding from oesophageal varices and in 1933 Walters suggested that it might be advisable to inject a sclerosing solution into the varices at the time of ligation of the coronary vein.

In the discussion of the paper, Dr Edwin J. Grace states that in 1938 he injected 8 ml of sodium morrhuate into the coronary vein of a 53 year old male who had "massive oesophageal haemorrhage secondary to liver damage". This does not predate Crafoord and Frenckner's work. Moersch states in his paper that Pemberton (who was not a co-author of the paper) and he had thought of doing this procedure as early as 1933, however they thought it prudent to first demonstrate its safety and efficacy in the experimental laboratory.

They attempted to produce oesophageal varices in dogs, but only managed to produce "enlarged vessels over the thoracic wall" and were therefore reluctant to attempt this procedure on a human patient. After the report of Crafoord and Frenckner appeared, Moersch felt justified to now attempt the procedure. The patient was a 30 year old male with recurrent gastrointestinal haemorrhage, due to cirrhosis of the liver. Dr Walters referred the patient to Dr Moersch, who performed oesophagoscopy under local anaesthesia and found large varices at the lower third of the oesophagus which were almost obstructing the lumen. He injected 0.5 ml of 2.5% sodium morrhuate and states "much to my surprise, the procedure was accomplished with practically no bleeding". He repeated the procedure three more times at four day intervals and injected 1 ml of 2.5% sodium morrhuate at each procedure. The changes to varices which he describes is noteworthy. "The vessels that had been injected lost their bluish color and became yellowish gray."

The first proponent of sclerotherapy in Great Britain was Ronald Macbeth, a clinical lecturer in otolaryngology at the University of Oxford. He first described his technique and results of nine cases at the Fourth International Congress of Otolaryngology in 1949. Subsequently he published his experience of 30 cases in 1955 in the British Medical Journal.

The technique which he employed was to give the patient a general anaesthetic and then he used the largest Negus oesophagoscope which could be passed, and injected the varices with a long needle carrier mounted upon a bayonet type fitting which locked onto a 10 ml or 20 ml syringe. The sclerosant used was 5% sodium morrhuate and he injected 3 ml to 4 ml into each venepuncture and injected six varices per session. There were 14 cases with liver damage which had been shown biochemically, whose age varied from 5 to 72 years and of note is that 50% were dead within one year. In contrast, out of the 16 cases without "liver damage", 14 were "alive and well", although the mean follow up was not stated in this paper.

An interesting point in the paper was that in the follow up of one of these patients Macbeth notes that the patient was alive at 11 years, which implies that Macbeth must have started his sclerotherapy as early as 1944. Macbeth stated that patients could live normal lives. However he found the varices recurred and insisted that the patients be examined by oesophagoscopy once or twice a year indefinitely. He also advised that a hydrostatic bag be placed in the oesophagus post sclerotherapy for 2 hours if the varices injected were profuse in number or large in size. Macbeth stated that splenectomy was obligatory as adjunct therapy as it "makes the veins more readily controllable."

Despite these pioneering efforts, sclerotherapy failed to become a standard recognised modality of therapy, probably for a variety of reasons, one of which was the popularity of portocaval shunting. As the results of prospective portocaval shunt trials became available, it was apparent that these operations had a high morbidity and mortality and did not greatly improve long term survival (Larson, 1986).

Johnston and Rogers reported on 117 patients who had sclerotherapy with good results in 1973. In 1975, Bailey described a modification of the rigid Negus oesophagoscope which he demonstrated while in Cape Town, and this prompted Terblanche et al to set up the first prospective controlled trial in August, 1975. Their initial report in 1979 was encouraging. However, in 1983 they failed to show improved long term survival. Westaby et al in 1984 did however show an improvement. These latter authors, as well as Pacquet's paravariceal methods (1983), can be considered as the reason for the resurgence of the role of sclerotherapy.

1.4 SCLEROTHERAPY TECHNIQUE

1.4.1 Intravariceal vs Paravariceal Injection

Paravariceal injection was first described by Wodak in 1960 in the German literature (Terblanche et al, 1990).

Pacquet subsequently reported on a large series of patients whom he treated using paravariceal injection of polidocanol with excellent results.
The aim of paravariceal injection in the acute stage is to compress the varix. The subsequent inflammation is thought to help embed the varix in the wall as well as thicken the overlying oesophageal mucosa.

The technique of intravariceal injection is more widely practised. The original description in 1968 by Blenkinsopp, a pathologist from St Mary's Hospital, laid down the foundation for this technique. He showed that paravenous injection of sclerosant in a rat's tail vein had no impact on the flow and that intravenous injection was required.

Although most studies are divided into intravariceal and paravariceal injections, in my experience, even when one aims intravariceally, this is often accompanied by some degree of paravariceal injection as well. This is confirmed by Rose et al, (1983) who showed that in their hands this occurred frequently. Grobe et al, (1984) used venography during endoscopic sclerotherapy and found similar results.

Paravariceal injection, purposefully or inadvertently, is associated with a higher local complication rate. This is discussed in greater detail at a later stage of this thesis. Kitano et al (1987) has published a series where they advocate a combined technique. Most series that are reported use an intravariceal method (Sivak et al, 1990). The technique employed in both trials in this thesis was intravariceal.

1.4.2 Instruments

There are two instruments available, i.e. rigid and flexible endoscopes. A prospective trial comparing the two methods has been reported (Bornman et al, 1988). They found that the rate of serious complications was higher in the rigid endoscopy group. A disadvantage of the rigid technique is that general anaesthesia is required. While some endoscopists might feel that this is advantageous in that the risk of aspiration is reduced, others feel that this is offset by the dangers of anaesthesia in patients with poor liver function (Sivak et al 1990). For both trials in this thesis, flexible endoscopy was used. The use of an overtube for compression is controversial and is discussed in Chapter 2. An overtube was not used in either of the clinical trials.

1.5 THE SCLEROSANT

Various sclerosants are available. However, their mechanism of action is poorly understood (Sivak et al, 1990). The sclerosant used in this thesis was Sodium Tetradecyl Sulphate (S.T.D.). The reason for choosing this sclerosant included the pioneering work by Blenkinsopp, who showed it to be superior to other sclerosants in a rat model.

The availability of the sclerosant was an important factor. In South Africa one of the only other sclerosants which is available is ethanolamine oleate and although Kitano et al (1987) and Terblanche et al (1990) prefer this sclerosant, Sivak et al (1990) still recommend the use of S.T.D.

A third factor for choosing S.T.D. was its viscosity and colour. S.T.D. is a clear non-viscous solution as compared to the yellow viscous ethanolamine oleate. This facilitated the use of various concentrations where the patient, the endoscopy assistant and the endoscopist were unaware as to what concentration of sclerosant was being injected. Ethanolamine oleate is extremely difficult to inject.

Therefore if it had been chosen it would not have been possible for the investigators to be blinded as to the concentration of the sclerosant.

The final reason for choosing S.T.D. is that S.T.D. is a single substance rather than a mixture of oils (ethanolamine oleate) and therefore it would be more amenable to modification should this prove necessary. S.T.D. is an anionic detergent. Its structure is shown in Figure 1.2. It is supplied as 3% mass per volume and is preserved with benzyl alcohol 2% m/v. It is a sterile aqueous solution and is buffered for a pH of 7.6.

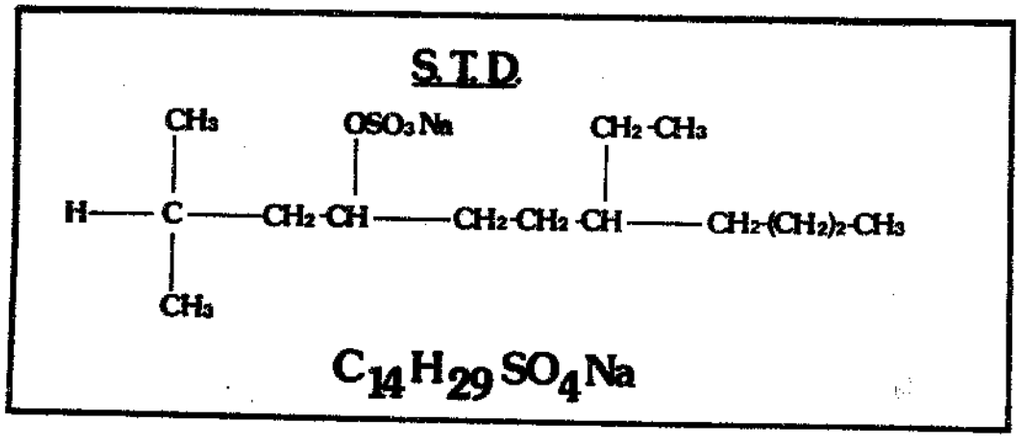


FIGURE 1.2 : The chemical structure of Sodium Tetradecyl Sulphate.

1.6 ROLE OF SCLEROTHERAPY IN CONTROLLING THE ACUTE BLEED

Sclerotherapy has been shown to control varices in 95% of episodes (Terblanche et al, 1981) and their findings have been confirmed by numerous workers. A problem has been that various authors use different techniques as well as sclerosants. Terblanche's findings were similar to those of Johnston, who in 1973 had a 93% success rate in stopping the acute bleed.

The rebleed rate would appear to be in the region of 13 - 30%. The high mortality would appear to correlate well with the degree of underlying liver disease, viz. Child C has a much worse prognosis than Child A (Larson, 1986). Larson et al showed in 1986 that patients who underwent sclerotherapy had a significantly lower transfusion requirement than those who did not.

Sivak et al (1981) reported on 22 patients and showed a marked decrease in transfusion requirements prior to initiation of sclerotherapy in theses patients. A study performed on 52 patients by Cello et al (1984) where patients were randomized to sclerotherapy or shunt surgery showed that sclerotherapy patients had shorter hospitalisation periods and transfusion requirements than those patients who had shunt surgery. However, hospitalisation periods for rebleeding was higher in the patients who had sclerotherapy.

In a study from the Massachusetts General Hospital, von Ryll Gryska et al (1985) used a combination of thrombin, cephalothin and ethanolamine oleate to sclerose 113 patients with varices. Sclerotherapy was performed for acute bleeding varices in the majority of their patients. Their initial success rate was 95%.

An important prospectively randomised trial showed a marked benefit of sclerotherapy versus tamponade (Pacquet et al, 1985). In this trial 43 cirrhotic patients were randomised to either sclerotherapy or Sengstaken-Blakemore tube for actively bleeding oesophageal varices. Long term survival, as in most studies, was related to Child classification on admission. However, efficacy in stopping the acute bleed and initial mortality was significantly improved in the sclerotherapy group. Despite all the claimed benefits for the acute bleed, one should be aware that as many as 75% of patients will have stopped bleeding at the time of initial endoscopy prior to sclerotherapy (Shields, 1991).

1.7 ROLE OF SCLEROTHERAPY IN CONTROLLING RECURRENT VARICEAL HAEMORRHAGE

It appeared logical that if sclerotherapy was efficacious in controlling acutely bleeding oesophageal varices, long term management would obliterate and reduce recurrent haemorrhage. The first controlled randomised trial of sclerotherapy by Terblanche (1983) showed that while the sclerotherapy group had significantly fewer rebleeding episodes, their overall survival rate at 1 and 5 years was not significantly different from the control group. MacDougall et al, in 1982 showed a statistically significant improvement in survival at 1 year and of note is that there was an increased incidence of death due to bleeding in the control group.

The major study by Westaby et al (1985) showed a statistically significant improvement in survival of patients who had received sclerotherapy. There was also a decrease in both the total number of patients who rebled as well as the number of episodes. This study also showed improved survival for each of the Child's grades of severity. Soderlund et al (1990), also showed a decreased risk of rebleeding. Sivak et al (1990), feel that despite the above studies, recurrent bleeding still occurs in 30-60% of patients.

The fact that Child's classification plays an important role is borne out in numerous studies (Von Ryall Gryska et al, 1985; Pacquet et al, 1985; Westaby et al, 1985).

In a meta-analysis which examined several controlled clinical trials, the results showed an improvement in long term survival attributable to serial sclerotherapy (Infante-Rivard et al, 1989). The question of long term survival remains controversial. The Copenhagen study (1984) which randomized two groups into medical and sclerotherapy showed no difference in the long term survival. An important study by Sauerbruch et al, (1985) showed that the mortality was directly related to Child's Classification. Child's A 1 year survival was 100% vs 38% for Child's C.

Not only does Child's classification play a role, but perhaps more importantly the fact that patients Child's classification is not static but rather dynamic and is based on the underlying hepatic pathology. It has been suggested that sclerotherapy has been overutilized at some centres at the expense of other therapeutic modalities including shunt surgery and pharmacotherapy (Terblanche, 1990; Shields, 1991). It would appear that the underlying hepatic pathology must be the major determinant in long term survival of patients whatever modality is used.

1.8 COMPLICATIONS OF SCLEROTHERAPY

Sclerotherapy has been associated with numerous complications. The following is a review of some of the important complications which have been documented.

1.8.1 Systemic Complications

An important systemic complication is the effect of the sclerosant on the pulmonary circulation. One of the most common thoracic complications is pleural effusion. The incidence would appear to correlate with the total volume injected and the volume injected per site but not with either the type of sclerosant or the total volume per session (Edling et al, 1991). Roentgenographic findings other than effusions include deviation of the azygo-oesophageal reflection into the right hemithorax, thickening of the posterior wall of the bronchus intermedius, an indistinct periaortic stripe and subsegmental atelectasis. The effusion when aspirated and analysed is normally a sterile exudate (Edling et al, 1991). Bacon et al (1985) also showed radiological evidence of pleural effusions in numerous patients.

There is evidence that the sclerosant embolises to the lung in numerous patients. De Peuy et al, in 1988 performed a study where they showed evidence of embolization to the lungs at 2 hours in 60% of cases. Acute respiratory failure was documented by Monroe et al (1983).

Vallgren et al (1988) performed a study on seven sheep to determine the effect of sclerosing agents. They tested ethanalamine oleate and S.T.D. and injected them intravenously. Both agents caused a severe fall in total respiratory compliance and arterial oxygen tension. There was marked trapping of platelets in the lungs. They concluded that both sclerosing agents cause severe lung injury in sheep if given in corresponding doses of 20-50% of what is normally used for sclerotherapy in human patients and postulate that the mechanism is based on increased microvascular permeability. Another interesting paper demonstrated that sclerosants caused pulmonary hypertension in sheep (Hammond et al, 1985). These workers found that the raised pulmonary pressure could be obviated by pretreating the sheep with indomethacin which suggests that the raised pressure is due to sclerosant-mediated prostaglandin release. Sigurdsson et al (1989) confirmed the above by demonstrating that aspirin prevents pulmonary damage in sheep given ethanolamine oleate.

Connors et al (1986) injected sclerosant containing Technetium labelled albumin microspheres and demonstrated that in their series only 20% of the injected sclerosant reached the pulmonary vasculature.

They showed no changes in the pulmonary diffusion capacity using a carbon monoxide technique. Endoscopy can cause relative hypoxia in nearly half the patients undergoing the procedure (Barkin et al, 1989). Korula et al (1984), however, demonstrated transient deterioration in pulmonary function after sclerotherapy.

The effect of sclerotherapy on cardiovascular haemodynamics was assessed by invasive monitoring in 8 patients (Bailey-Newton et al, 1985). They demonstrated a statistically significant increase in pulmonary vascular resistance, although the elevated levels were still within normal limits.

Other pulmonary complications described include bronchitis (Barsoum et al, 1982), adult respiratory distress syndrome due to massive chylothorax (Gertsch et al, 1983) and a fatal right lower lobe collapse (Barsoum et al, 1982).

Another interesting systemic complication is haemorrhage from other sites. Foutch and Sivak (1984) demonstrated 3 cases which bled from colonic varices after endoscopic sclerotherapy. Keane et al (1986) reported on massive bleeding from rectal varices following repeated injected sclerotherapy of oesophageal varices.

Fry et al (1988) reported on a 78 year old patient with haematochezia which occurred post sclerotherapy of oesophageal varices. They found, at laparotomy, that it was due to adhesion related varices at the site of a previous hysterectomy. They did not comment on the sclerosant used for the oesophageal varices.

Distal sites of thrombosis have been reported. Goodale et al (1982) described superior mesenteric vein thrombosis in 2 patients. The sclerosant which they used was 5% sodium morrhuate. In the same year Barsoum et al, described a patient who developed portal vein thrombosis post sclerotherapy using ethanolamine oleate. This resulted in recurrent haemorrhage which proved to be fatal. A tragic case of spinal artery thrombosis which occurred in a 4 year old child after intravariceal injection of 5% sodium morrhuate was described by Seidman et al in 1984. This resulted in subsequent paraplegia. More recently, Ng et al (1988) described a case of digital gangrene which occurred post sclerotherapy.

Duodenal varices have been described as a complication after endoscopic sclerotherapy (Eleftheriadis, 1988). The cause of this complication is obscure. Gastric varices may increase in number and size after sclerotherapy (Eleftheriadis, 1988).

However Sarin et al (1986) and MacDougall et al (1982) reported that the recurrence of oesophageal varices was not associated with gastric varices.

Congestive gastropathy is a common finding and occurs in a severe form in 55% of patients with varices. It has also been documented in one trial to occur in 79% of patients who received sclerotherapy (D'Amico et al, 1990). In their study, they felt that recurrent bleeding was in some cases due to the gastropathy. This is at variance with other studies (Westaby et al, 1985; Kitano et al, 1987), where recurrent bleeding was associated with recurrence of varices. The pathogenesis of portal hypertensive gastropathy is controversial. Decreased mucosal blood flow, congestion and ectasia, decreased pepsinogen I, increase serum gastrin and increase mucosal prostaglandin E_2 levels have all been incriminated (Blackstone, 1991). Chung et al (1988) described a technique of endoscopic measurement of gastric blood flow using laser Doppler velocimetry. Although gastric mucosal blood flow was elevated in patients with portal hypertension, the mucosal flow was not altered after sclerotherapy.

1.8.2 Local Complications of Sclerotherapy

1.8.2.1 Oesophageal Stricture

This is a reasonably common complication occurring after sclerotherapy. Haynes et al, (1986) compared a group of patients who developed stricture post S.T.D. injection, with a group in whom this did not occur. They could find no differences in either the total volume or number of injections between the two groups. Oesophageal dilation was successful in all cases. The use of intentional paravariceal injection has a high stricture rate (Kjaergaard et al, 1982).

1.8.2.2 Oesophageal Perforation

Oesophageal perforation has been reported as a complication of sclerotherapy (Perino et al, 1987).

1.8.2.3 Oesophageal Ulceration

The incidence of ulceration would appear to vary directly with the time interval of endoscopy post sclerotherapy. Sarin et al (1986) showed that 94% of patients, when they were endoscoped 24 hours after the procedure, developed ulceration after sclerotherapy with absolute alcohol. This figure dropped to 69% at one week and at 3 weeks the figure was 12.5%.

1.8.2.4 Oesophageal Motility

Decreased oesophageal motility and acid clearance have been associated with sclerotherapy, although the literature is controversial (Sivak et al, 1990).

1.8.2.5 Chest Pain

Numerous patients complain of chest pain post sclerotherapy. This pain has been thought to be due to a chemical mediastinitis but this remains to be proven (Sivak et al, 1990). Gebhard et al (1982), on the basis of barium swallows, suggested that it was due to oesophageal spasm.

CHAPTER 2

2. THE SYSTEMIC EFFECTS OF 3% SODIUM TETRADECYL SULPHATE ON COAGULATION

2.1 INTRODUCTION

Although the systemic effects of sclerotherapy on coagulation have been previously reported, no trials to date have assessed the systemic effects of S.T.S. on the coagulation system and liver function.

Gardner and Brooks in 1982 performed a study on 11 patients at Emory University Hospital. They injected a combination of 2 ml of 5% sodium morrhuate followed by 2 ml of an 8 ml mixture containing 1 ml (100 units) of thrombin, 2 ml of cephalothin sodium concentration (1 g/5 ml) and 5 ml of 50% dextrose into each varix. A balloon for proximal dilation was used. At 7 a.m. on the day of sclerotherapy, the haematocrit, haemoglobin, platelet count, prothrombin time, partial thromboplastin time, fibrinogen, fibrin split products, Factor V and Factor VIII were analysed (approximately 16 hours after the completion of the procedure). They found no statistical differences in any of the parameters measured.

They concluded that the sclerosant combination they were using had no systemic effects and postulated that the reason for the lack of systemic effects was that they used an overtube.

This tube consisted of a balloon cuff which was attached to the flexible endoscope proximal to the instrument tip and inflated prior to sclerosant injection and for 1 minute afterwards. This was performed in an attempt to retard cephalic spread of the sclerosant.

The problem with this study was the time interval for testing of clotting parameters. They only tested the patients approximately 16 hours post sclerotherapy and would therefore have missed any significant changes which would have occurred during this crucial time period. The time of approximately 16 hours would fail to detect a transient coagulation defect. One would expect that if a coagulopathy such as a diffuse intravascular coagulopathy (which is what the authors were specifically looking for) was present 16 hours after the injection, then these patients would have had marked clinically apparent signs of coagulation disturbances. The authors did not document or discuss the clinical appearance of their patients post sclerotherapy. Their technique was intravariceal and they used an overtube.

The use of an overtube is controversial. Grobe et al (1982) showed the flow of the varices was cephalic in 50% of patients with or without balloon compression and that in 15% the flow was as caudal with or without compression. In 35% the sclerosant accumulated locally. which suggests either paravariceal injection or no flow in the varices injected.

However, 3 hours later, there was no residual contrast material in the chest or abdomen suggesting that the latter was more likely.

The sclerosant which was used in this trial was 5% sodium morrhuate mixed with an equal volume of 60% renograffin. A total of 10 - 20 ml was injected at each session. The authors concluded that balloon compression was probably unnecessary during sclerotherapy.

Bernardi et al, a year later in 1983, criticized Gardner's paper because they felt that the time interval was too lengthy and any transient coagulopathy would be missed. They evaluated the coagulation profiles in 10 patients with liver cirrhosis before, 30 minutes after, and 18 hours after elective injection of oesophageal varices. They injected 5% ethanolamine oleate . Only 3 parameters were measured; the platelet count, Owren's normotest and plasma fibrinogen. All these parameters were significantly impaired at 30 minutes. The platelet count was reduced, as was fibrinogen and Owren's normotest. The authors suggested platelet consumption and coagulation factors occur shortly after injection sclerotherapy using 5% ethanolamine oleate.

Baele et al in 1984 published an abstract where they described the effects of injecting 1% polidocanol on the haemostatic system. They injected 12 patients with liver cirrhosis.

They measured the following parameters; the activated partial thromboplastin time, the prothrombin time, Factors II, V, VII, VIII, IX, X, fibrinogen, plasminogen, antithrombin III and alpha-2 antiplasmin and fibirin(ogen) degradation products. These tests were performed before, 10 and 60 minutes after sclerotherapy in 12 patients.

They found no significant changes in any of the parameters tested except for one patient whose fibrinogen degradation products became positive. They concluded that polidocanol did not activate the coagulation pathway.

Musso et al in 1987 published a letter where they assessed 25 patients who were sclerosed with 1% Polidocanol for oesophageal varices. The following parameters were measured immediately before, and 10 minutes, 60 minutes, 360 minutes and 24 hours after sclerotherapy, viz. platelet count, prothrombin time, thrombin-coagulase time, fibrinogen, fibrin(ogen) degradation products, Factor XII and plasma prekallikrein levels.

Their results showed a statistically significant drop in Factor XII and plasma prekallikrein levels at 10 minutes which had returned to normal at 60 minutes. The authors concluded that Polidocanol was able to activate the contact phase of coagulation which implies that the reason for the low factor XII is consumption. Kang et al in the same year (1987) published a trial in the British Journal of Surgery. They evaluated 20 patients with oesophageal varices.

These patients underwent endoscopic injection sclerotherapy with 5 percent ethanolamine oleate.

The following parameters were assessed: Activated partial thromboplastin time, the Normotest, plasma fibrinogen, fibrinogen degradation products and complete blood cell count. Fibrinopeptide A and fibrinopeptide B β 15-42 were also measured. The time interval used for sampling was before the sclerotherapy, one hour after the sclerotherapy, 1 day, 2 days and 3 days after sclerotherapy.

Their findings showed a statistically significant decrease in prothrombin time at 1 day after sclerotherapy although this did not appear to be of clinical significance. There was a significant leucocytoses at 1 and 2 days post sclerotherapy and the plasma fibrinogen had gradually increased at day 3. They also showed a statistically significant increase in plasma fibrinopeptide A and fibrinopeptide B β 15-42 at 1 hour and 1 day post sclerotherapy. Fibrinopeptide A is formed after being liberated from fibrinogen by the action of thrombin and fibrinopeptide B β 15-42 is liberated by plasmin. These tests therefore were accurate in assessing and confirming a transient activation of coagulation and fibrinolysis after sclerotherapy. The authors however do note that this might be a secondary phenomenon that they are measuring due to the inflammatory responsive rather than a direct action of the ethanolamine oleate. An alternate explanation might be that the fibrinopeptides are released from thrombus which has formed on the ethanolamine oleate induced damaged endothelium.

2.2 AIM

The aim of the trial was to determine the systemic effects of the sclerosant, sodium tetradecyl sulphate (S.T.D.), with particular reference to the coagulation system. At the time that the study was conducted, S.T.D. (3% m/v) was one of the sclerosants in routine usage at Baragwanath Hospital. Sclerotherapy was not used as a primary modality for controlling the acute bleed in patients with oesophageal varices. It was used as a treatment for prevention of rebleeding in patients who had presented with bleeding at admission or who had a history of a prior oesophageal variceal haemorrhage. Patients who presented with an acute variceal bleed were treated in the first instance with a Sengstaken-Blakemore tube. Sclerotherapy was only performed one week after the incident in line with policy at Baragwanath Hospital at the time. The follow up of patients was and remains extremely poor at Baragwanath Hospital, so the study was confined to the acute effects of the sclerosant.

2.3 PATIENTS

Fifteen adult patients with portal hypertension from Baragwanath Hospital were included in the trial. All patients gave informed consent and the trial was approved by the Human Ethics Committee of the University of the Witwatersrand. The mean age of the patients wa 9 years (range 30-69 years). There were 12 male and 3 female patients.

The only criterion for inclusion in the study was any patient who presented with oesophageal varices who had a history of a prior variceal bleed.

All fifteen patients had liver biopsies to obtain histological evidence of the aetiology of their cirrhosis. Of these, 13 patients had alccholic liver cirrhosis, 1 had schistosomiasis and in 1 patient the cause was undetermined.

The Child-Pugh classification of the patients was as follows 9 of the patients were Child's A, 4 were Child's B, and 1 was Group C.

The patients were all sedated with 5mg of midazolam.

2.4 MATERIALS AND METHODS

All patients had a full upper gastrointestinal endoscopy performed using a forward viewing Olympus flexible gastroscope to document any concurrent pathology which might have been responsible for the bleed. The patients then had one or more varices injected. The injection was aimed intravariceally although is probable that some of the sclerosant was probably injected paravariceally. An oversheath was not used. The agent used was sodium tetradecyl sulphate 3 m/v, preserved with benzyl alcohol 2% m/v. The agent was supplied by MPS Laboratories (Pty) Limited. The amount of sclerosant injected varied from 6 to 12 mls, with a mean volume of 8.6 mls injected.

All patients had blood drawn immediately prior to sclerotherapy, immediately after sclerotherapy, and 30 minutes, 60 minutes and 24 hours post sclerotherapy. This was performed to ensure that any transient abnormalities were not missed .

These specimens were submitted for analysis of the following parameters:

prothrombin time, partial thromboplastin time, fibrinogen, fibrin(ogen) degradation products (F.D.P.), Factor VIII, full blood counts and liver function tests.

The methodology employed in the prothrombin time, partial thromboplastin time, fibrin(ogen) degradation products, and factor VIII assays is identical to the methods described in the section on the in vitro trials. The full blood counts were performed on a Coulter S Plus Analyser and the liver function tests were assayed on a Instrument Laboratory 503 Abbot 100 machine

2.4.1 Statistics

The statistical analysis was done by the Institute for Biostatistics of the South African Medical Research Council.

The many-one tests, ie. comparing the pre-sclerotherapy values individually to all the post-sclerotherapy values, were performed by employing Wilcoxan's signed rank test using the Bonferroni adjustment for multiple comparisons.

2.5 RESULTS

2.5.1 Clinical:

All the patients experienced retrosternal chest pain immediately after sclerotherapy. All the patients were hospitalized overnight for observation. Two of the patients complained of mild dyspnoea post sclerotherapy. None of the patients had a haematemesis during this period. There were no other documented complications. Follow up on these patients is not available.

2.5.2 Laboratory:

The white cell count was significantly decreased at 60 minutes ($p=0.0115$), and significantly increased 24 hours post sclerotherapy ($p=0.024$) [Figure 2.1]. There was a statistically significant decrease in fibrinogen from 2.92 g/l to 2.68 g/l 60 minutes after sclerotherapy as compared to before ($p=0.0355$) [Figure 2.2]. In three patients this was associated with a mild elevation of the fibrin(ogen) degradation products. There was a trend, although not statistically significant, for the prothrombin index to decrease at 60 minutes after sclerotherapy [Figure 2.3].

The remaining parameters of the coagulation profile, viz. Factor VIII levels and the activated partial thromboplastin time were not significantly altered.

The haemoglobin, mean cell volume and haematocrit and platelet counts did not show any statistically significant alterations. The platelet count showed a trend to decrease at 30 minutes post sclerotherapy. However, it was not statistically significant.

The liver function tests, which included total protein, direct bilirubin, total bilirubin, albumin, globulin, alanine transaminase, aspartate transaminase, gamma glutamyl transaminase and alkaline phosphatase were all unchanged.

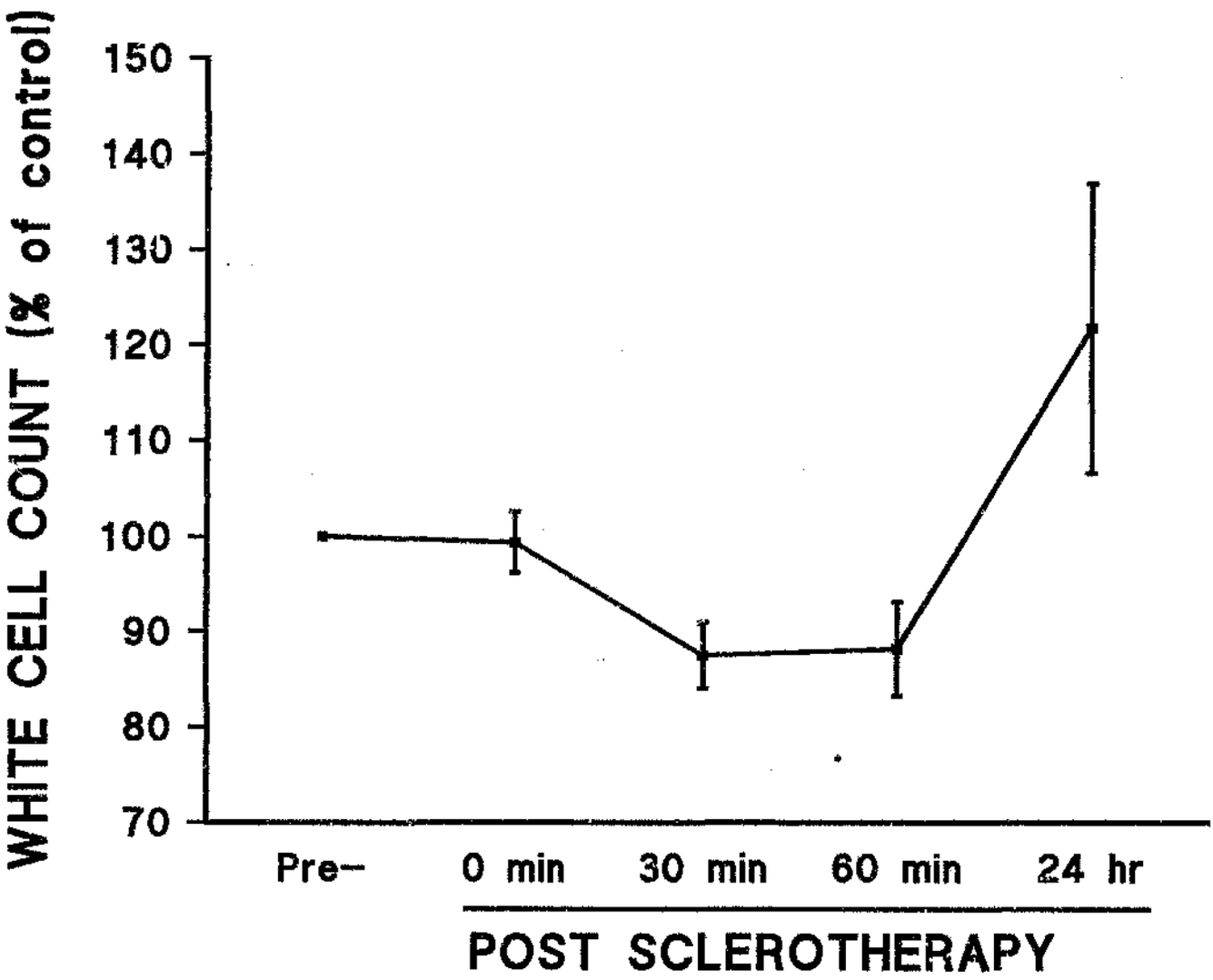


FIGURE 2.1

The in vivo effect of intravariceal injection of S.T.D. on the white cell count. (Mean ± SEM)

At 60 min p=0.0115 as compared to Pre–.
At 24 hr p=0.024 as compared to Pre–.

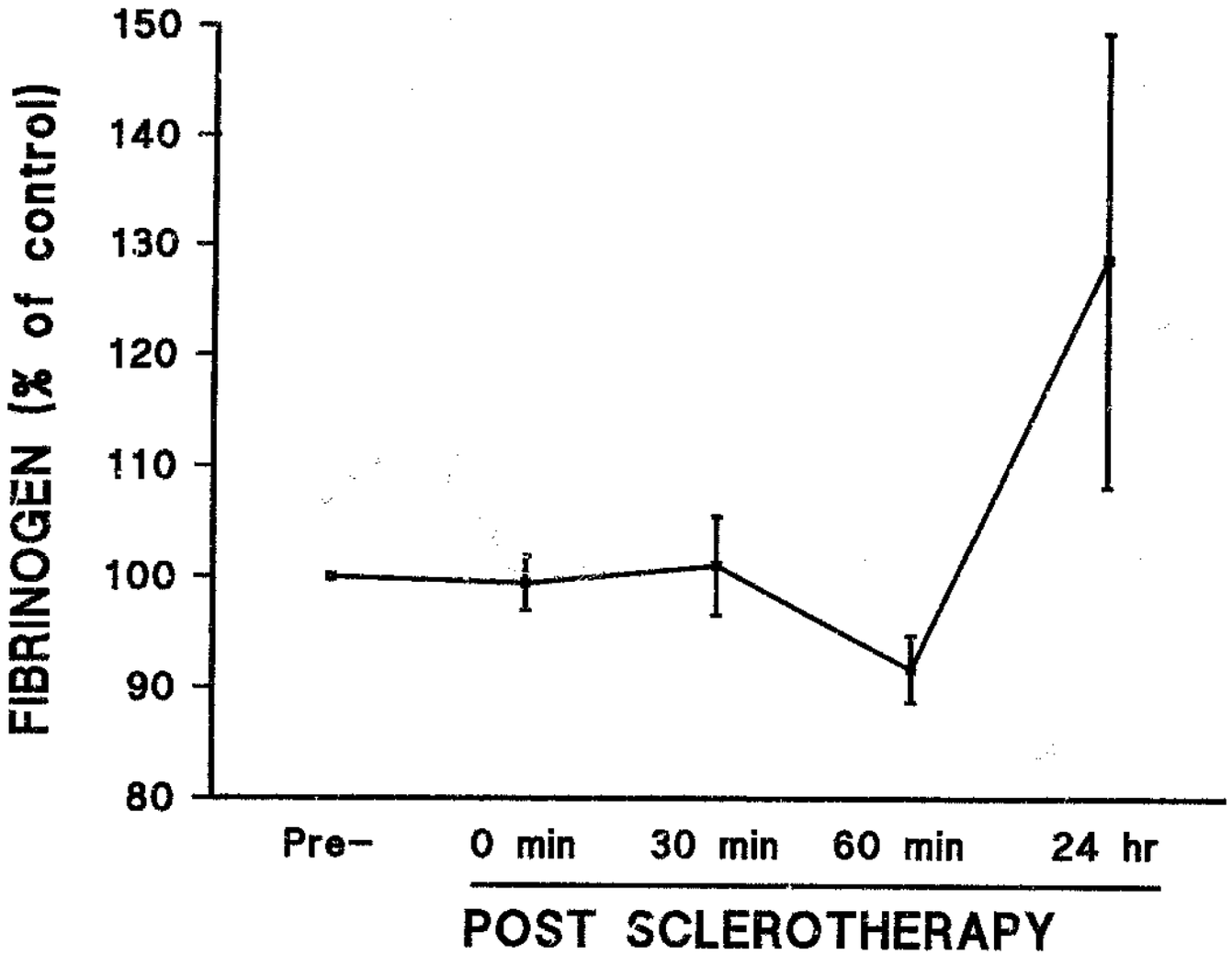


FIGURE 2.2

The in vivo effect of intravariceal injection of S.T.D. on fibrinogen levels. (Mean ± SEM)

At 60 min p=0.035 as compared to Pre–.

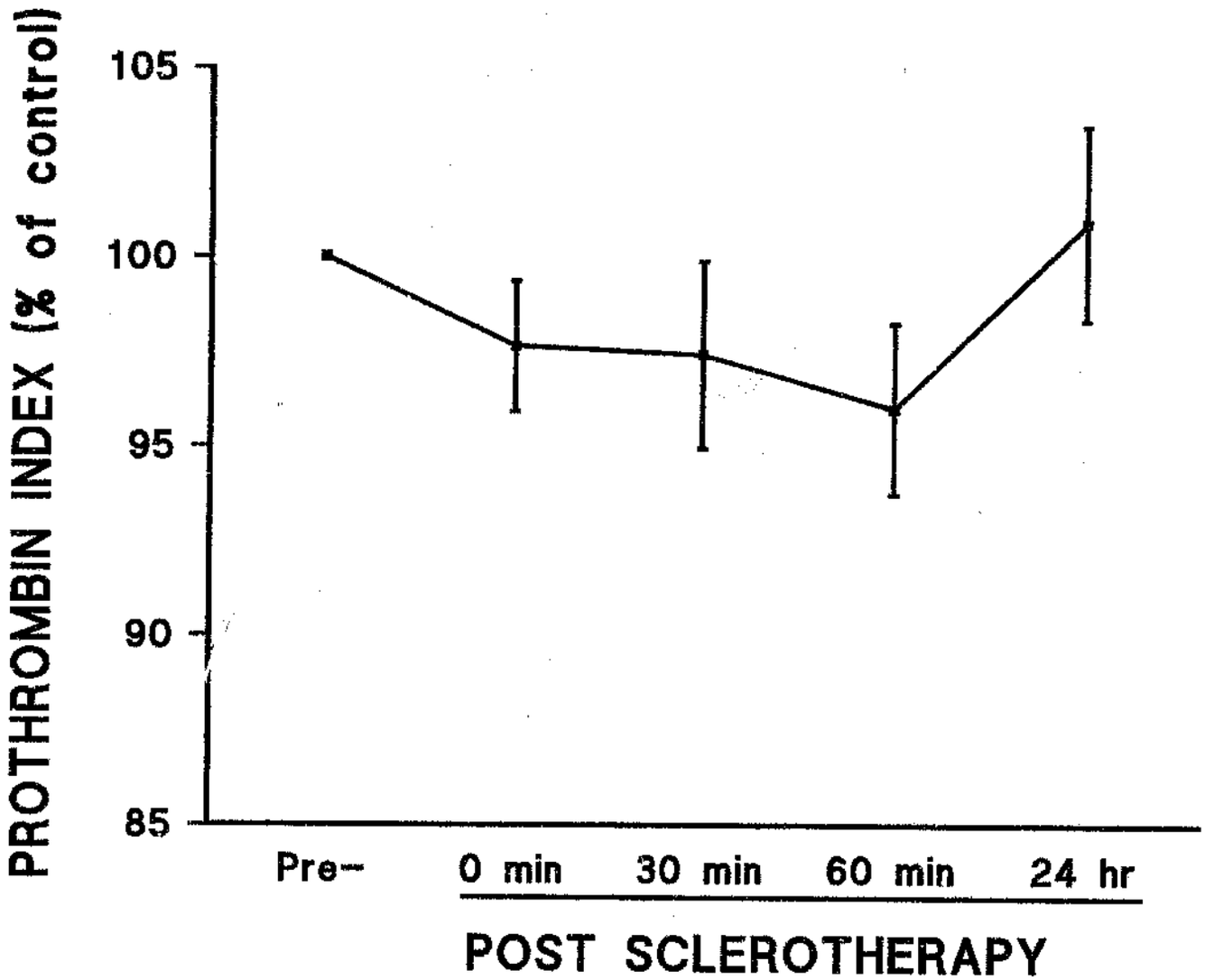


FIGURE 2.3

The in vivo effect of intravariceal injection of S.T.D. on the Prothrombin Index.

(Mean ± SEM)

2.6 DISCUSSION

In the present study 3% m/v S.T.D. was demonstrated to have a transient systemic effect on coagulation. There was a statistically significant decrease in the level of fibrinogen. While this did not result in an acute bleeding diathesis one would not have expected this to occur as the fibrinogen concentration did not drop below a level which would result in a bleed. The same argument holds true for the trend for the prothrombin index to decrease. The question is whether the S.T.D. destroyed these factors directly or whether they were consumed. The in vitro work presented later suggests that at the volume and concentrations injected, the former is unlikely.

The transient drop in the white cell count which was followed by a leucocytoses is interesting.
Leucocytes have been shown to play a role in thrombosis. Belch (1985) in a review article states that the leucocyte has the ability to damage endothelial cells by means of free radicals. The endothelial cells then produce less prostacyclin and the breakdown of endothelium derived relaxing factor is increased (McCord, 1985). Furthermore neutrophils interact with platelets and stimulate aggregation (Canoso et al, 1974).

The converse is also true, i.e. that platelets can stimulate leucocyte activation (MacLouf et al, 1982). The drop in the leucocyte count was probably due to margination and sequestration.

This was followed by a rebound leucocytoses which occurs as a result of cell release from the marrow.

The above phenomena are analogous to the events which occur post endotoxin administration to human subjects (Golde, 1990). Other reasons for the rebound leucocytoses include a transient bacteraemia which sclerotherapy can induce, and pyrexia which is thought to be caused by the chemical phlebitis or the acute inflammatory response (Schuman et al, 1987).

The site of the sequestration of white cells which occurred was probably in the lungs. Sclerotherapy is a well documented cause of acute adult respiratory distress syndrome, the basis of which is postulated to be endothelial damage (Stroncek et al, 1985). In a large proportion of patients there is rapid flow of sclerosant from the varices to the pulmonary circulation (Grobe et al, 1982). This would also explain the transient fall in fibrinogen levels, as well as the elevated fibrin(ogen) degradation products which was found in three patients.

The findings of this study showed that sclerotherapy with S.T.D. did have systemic effects on the coagulation system and suggested that modifying the dose might be a useful adjunct in limiting the above effect.

CHAPTER 3

3. PLATELETS

3.1 INTRODUCTION

The in vitro effect of sclerosants on platelet function has not been adequately studied. Stroncek et al (1985) demonstrated that sclerosants cause haemolysis and that this haemolysate could induce platelets to aggregate. These workers suggested that the aggregation was due to the red cell membrane, which is a documented agonist capable of causing platelet aggregation. Yamaga et al (1989) showed in an in vivo trial that ethanolamine oleate depressed platelet aggregation as evidenced by a decreased response to ADP for 1 week after sclerotherapy.

The function of platelets has been reviewed extensively in recent years (Roth, 1991; Bloom, 1990; Ginsberg et al, 1988). The following is a selective review of platelet function, which, although not comprehensive, is relevant to the understanding of the mechanism of action of S.T.D. with particular reference to platelet aggregation.

3.1.1 Platelets and their Function - A Review

Bizzozero showed in 1882 that platelets accumulate at injury sites in small vessels and thus established the crucial role that platelets play in forming the haemostatic plug. Wright in 1906 described the origin of platelets. He discovered that they are produced by the megakaryocyte.

Platelets circulate in a resting phase and their survival is estimated to be approximately 8 to 12 days (Heyns et al, 1980).

3.1.1.1 Platelet Structure

One of the mechanisms which enables them to perform their function is their trilaminar platelet membrane. This membrane does not appear to be inherently different from other cell membranes on initial analysis. However freeze fracture analysis has shown that the platelet membrane is in fact different from other cells in that it is turned inside out (Zucker-Franklin D, 1990). Platelet membranes have numerous glycoprotein receptors which are summarized in Table 3.1.

Platelets contain organelles, including mitochondria but are anucleic. The organelles include the alpha granules and the dense bodies. Alpha granules contain ß thromboglobulin, fibrinogen, thrombospondin, fibronectin, platelet derived growth factor, platelet factor 4, and von Willebrand factor (Wencel-Drake et al, 1985), while dense bodies contain serotonin and probably ADP. The density of the granule would appear to be proportional to the amount of serotonin present (Tranzer et al, 1966).

TABLE 3.1

PLATELET MEMBRANE GLYCOPROTEIN	FUNCTION
Ia	Collagen receptor
Ib	vWF receptor
Ic	Fibronectin receptor
IIa	Complexes with Ia and IIc
IIb	vWF receptor Fibronectin receptor
IIIa	Vitronectin receptor Fibrinogen receptor
IV	Thrombospondin receptor
V	Thrombin receptor

Small vesicles contain lysosomal enzymes, viz. acid phosphatase, ß glucoronidase, cathepsin and arylsulfatase (Bentfield-Barker et al, 1982). Platelets contain a canalicular membrane system which is one of the mechanisms responsible for exocytosis of the granules. After the platelets have been stimulated by an agonist, the granules coalesce in the centre of the platelet together with canalicular cisternae (Zucker-Franklin, 1970).

3.1.1.2 Adhesion interactions between Cells and the vessel Wall

Cell adhesions are responsible for sealing off leaks which occur inside blood vessels. These adhesions require surveillance which is specific as well as selective and occur as a result of cell-adhesive molecules, also known as anchorins. These bond a site on one cell to a site on another cell or solid phase.

It is estimated in man that 1 trillion platelets survey 1 000 square meters. Should the platelets encounter a defect in the vessel wall, it will change its shape, adhere and spread on the surface and seal the leak by initiating a platelet plug. Pethica (1961) demonstrated various processes of intercellular adhesion.

The initial process involves membrane protrusions on a smooth surface which reduced electrostatic repulsion and thereby promotes adhesion. For example, a pseudopod protruding from a platelet is probably able to penetrate the electrostatic forces on the opposite surface. Another process involves the presence of polyvalent ions, e.g. calcium, which further reduce the electrostatic negative force. A final mechanism which he proposed is the presence of large molecules which can bridge a distance of greater than 10 Angstroms. This process is utilised in platelets, in that platelets contain and can bind fibrinogen which is 75 Angstroms long and 60 Angstroms wide and can therefore span a large area and overcome hydrostatic as well as electrical charges.

For platelets to be functional they require not only cellular adhesive molecules but also intercellular adhesive molecules. The cellular adhesive molecules may operate independently in some cases or with the help of an intercellular adhesive molecule.

An example of this is the glycoprotein IIb/IIIa complex, an integrin (Ginsberg, 1988), which has the ability to bind one platelet to another directly. However it can also bind to fibrinogen which then binds to the glycoprotein IIb/IIIa complex on another platelet. In this instance the fibrinogen is an intercellular adhesive molecule (Gogstad et al, 1982).

A third group of molecules act as substrate adhesive molecules. These substrate adhesive molecules are present in the subendothelium, an example of which is von Willebrand Factor. Platelets have specific receptors for the von Willebrand factor and this provides an anchor for the adhesive process to be stabilized.

All the intercellular adhesive molecules are produced in the alpha granule of the platelet. The identical intercellular adhesive molecules are produced in the endothelial cells and distributed in the ablumenal surface, thereby providing two "sticky" ends which will meet if the endothelium is damaged and the platelet is activated.

From an evolutionary point of view it is interesting to note that almost all adhesive proteins contain or bind the R-G-D tripeptide

sequence (arginine-glycine-aspartic acid) (Pierschbacher et al, 1984). It would appear that this particular sequence was crucial in the evolution of unicellular to multicellular organisms. In this regard it is of interest that the unicellular slime mould Dichtyostelium discoidaum acquires a R-G-D containing lectin when its food supply is exhausted. This then enables it to aggregate to other cells and survive (Springer et al, 1984). R-G-D sequences are essential for haemostasis and platelet function. However R-G-D sequences have acquired specificity and are not necessarily interchangeable, e.g. platelet aggregation does not occur in afibrinogenaemia (Inceman et al, 1966) and this defect is not corrected by addition of fibronectin. The specificity of R-G-D is thought to be determined by the amino acid sequence within the rest of the molecules as even a slight change in sequencing will cause major conformational changes (Berzofsky, 1985).

3.1.1.3 Fibrinogen binding to Platelets

Platelets from patients with congenital afibrinogenaemia are insensitive to ADP (Inceman et al, 1966). The platelet function can be restored by adding fibrinogen.

Solum et al (1965) showed that washed normal platelets failed to aggregate when either ADP or fibrinogen alone are added. The fraction of fibrinogen which is responsible has been determined by Niewiarowski et al (1977) to be the carboxy-terminal portions of the A-alpha chain and the gamma chain. It has been shown by Marguerie et al (1982) that 45 000 to 50 000 fibrinogen molecules can be bound per platelet in the presence of calcium.

3.1.1.4 The Fibrinogen Receptor and ADP Receptor

This receptor would appear to reside in the IIb-IIIa complex. Two classes of binding sites have been described by Kornecki et al (1981). They are h gh and low affinity receptors.

3.1.1.5 Platelet-Surface Interaction

Sixma et al, (1977) have performed morphological studies of the haemostatic plug and shown that after injury bleeding begins immediately. Within seconds the initial response is platelet adherence to the edge of the severed vessels. This results in the formation of a loose plug. After several minutes bleeding is halted by the plug becoming more consolidated.

The plug consists almost entirely of densely packed platelets which have no remaining granular context.

3.1.1.6 Endothelium and Platelets

The role of the endothelium is reviewed separately in this thesis. The endothelium has the ability to act as either a pro- or anti-coagulant. Most studies on the role of endothelium have been performed by damaging the endothelium and exposing the subendothelial layer (Kinlough-Rathbone et al, 1983).

3.1.1.7 Subendothelium and Platelets

Tranzer (1967) has shown that gaps between the endothelium and defects in the endothelium are promptly plugged by platelets.

Platelets adhere to either the basement membrane or other connective tissue elements. Stemerman et al (1972) performed experiments on primates using a balloon catheter to denude endothelium. Baumgartner (1973) used a similar technique on rabbits and showed that within 1 minute platelets were already deposited on the surface. Although these platelets had morphological alterations as evidenced by pseudopodia, they still had most of their granules.

Within 20 minutes the platelets had formed a continuous layer. This was under normal arterial flow conditions. In contrast, mural thrombi, peak at 10 minutes and disappear within 40 minutes. No fibrin is detected in either case. Platelets which are attached to the subendothelial surface secrete most of their alpha granules and 5-HT storage organelles.

Platelets spread rapidly on the subendothelium as compared to artificial surfaces (Baumgartner et al 1976). It is thought that spread platelets release granular material which promote adhesion (Weiss et al 1986).

3.1.1.8 Platelet Aggregation

Platelet aggregation is defined by Colman (1987) as that process by which platelets interact with one another to form a haemostatic plug. The process begins with adhesion of the platelet to subendothelial tissue consequent upon endothelial damage. Pseudopod formation of the platelet occurs as a results of collagen induced change.

The platelet also changes from a discoid shape to a spheroid shape, during which time it secretes its dense and alpha-granule contents.

The activated platelet forms a site for the inactivated platelet to collide against and platelet aggregates form. Fibrinogen binding is the crucial step for bridging from platelet to platelet and consequent aggregation.
Fibrinogen binds to the IIb-IIIa glycoprotein complex (Holmsen et al, 1979). Patients with Glanzman's Thrombasthenia lack this complex and therefore cannot aggregate their platelets.
For fibrinogen binding to occur the platelet must first be exposed to a chemical substance which will cause a latent receptor to be expressed on the cell surface. Examples of these substances include thrombin, adrenaline, collagen and certain prostaglandins. ADP is also an agonist and it is felt by most authors that ADP is the central mediating substance through which all the agonists work to expose the latent fibrinogen receptor. This action of ADP may be separate from the action of ADP which causes changes inside the platelet relating to decreasing cyclic AMP.

The aggregation is divided into 2 phases. The first involves exposing the fibrinogen receptor in response to an agonist. All the agonists, other than adrenaline, cause a structural change as evidenced by a shape change.

The second phase involves the binding of fibrinogen to platelets. This binding is initially reversible but later irreversible. The binding leads to aggregation.

3.1.1.9 A Model of Platelet Aggregation

The primary focus is a transmembrane protein which is the ADP or ATP binding site. The ADP binding site when activated is thought to cause a spatial reorientation of IIb and/or IIIa. This reorientation in the presence of calcium ions allows the IIb-IIIa complex to now be activated and therefore to act as the fibrinogen receptor. Adrenaline, collagen, prostaglandin and thrombin all require ADP binding to the IIb-IIIa receptor molecule, although their mode of action would appear to be mediated by binding to their own respective receptors which are probably not latent.

3.1.1.10 The Principle of Aggregometry

Born (1962) described a method of evaluation platelet function by using a platelet aggregometer. The basic principle is that platelets in suspension are relatively opaque. However, as they aggregate they cause a reduction in the total number of free particles in suspension and therefore the density decreases.

This change in optical density is measured with the platelet aggregometer. The aggregometer recognises two phases, viz., a primary aggregation wave and a secondary aggregation wave. The primary wave is present usually only at low concentrations of agonist and is reversible, the secondary wave is felt to be irreversible and is associated with secretion of the granular contents of the platelet.

3.1.1.11 Platelet Dysfunction in Liver Cirrhosis

Thomas et al published a trial in 1967 where they showed abnormal platelet aggregation in response to ADP in patients with liver cirrhosis. However, the technique which they used was a direct visual observation performed with a magnifying glass, hardly adequate technology by todays standards.

A large well performed study, which is often cited, using standard dual channel aggregometry, found that in the presence of a normal platelet count the platelet aggregation response to ADP, adrenaline, and collagen was normal (Stein and Harker, 1982). On the other hand, Desai et al (1989) showed a decrease in ADP induced aggregometry due to abnormal high density lipoprotein particles.

George and Shattil (1991) feel "it remains to be proved that platelet dysfunction due specifically to liver disease is a clinically important problem." Accordingly, the question of liver dysfunction in liver cirrhosis remains controversial.

3.2 AIM

The aim of this study was to evaluate the in vitro effect of S.T.D. on platelet aggregation.

3.3 METHODOLOGY

3.3.1 Platelet Aggregation

Venous blood was collected from four healthy volunteers. The blood was drawn with a 21 gauge butterfly needle and added to tubes in to ratio of 1 part 3.8% trisodium citrate, which was freshly made up, to 9 parts venous blood. The aggregation tests were performed in duplicate.

To determine whether platelet aggregation in patients with liver cirrhosis differs in response to ADP and STD, blood was taken from three patients (Mr E.N.; Mr P.M. and Mr L.M.) who all had liver cirrhosis (Child's B)

The tubes were then centrifuged in a Beckman Model TJ5 centrifuge at 130 g for 10 minutes. The platelet rich plasma was removed with a Gilson pipette using a plastic tip. The remaining sample was then centrifuged for a further 10 minutes at 1700 g. The platelet poor plasma was removed with a Gilson pipette. A platelet count was performed on the platelet rich plasma using a H*1 Technicon Auto Analyser.

The platelet count was then adjusted to 250 x 10^9l by addition of platelet poor plasma and 450 microlitres of the plasma with the adjusted platelet count was used. This plasma was pipetted into a siliconised glass tube followed by the agonist.

The following agonists were prepared (Table 3.2):

i) ADP from British Drug House which contains adenosine -5'- diphosphoric acid trilithium salt was made up to a stock concentration 15 mg/ml.

ii) Collagen was obtained from Hormon Chemie Munchan, GMBH and was diluted 1:10 with SKF Horm Buffer (Prod No 10500).

iii) Adrenaline was made up from May and Baker injection ampoules containing Adrenaline Tartrate equivalent to 1:1000 m/v adrenaline. These were diluted 1:10 with normal saline.

iv) Ristocetin Sulphate was obtained from H. Lunbeck A/S, Copenhagen. One hundred milligrams was dissolved in 6.6 ml of normal saline.

vi) Arachidonic Acid was made up to a stock concentration of 10 mmol/l.

TABLE 3.2

AGONIST	VOLUME ADDED	FINAL CONCENTRATION
Ristocetin	50 ul	1.5 mg/ml
ADP	50 ul	5 ug/ml
Adrenaline	50 ul	10 ug/ml
Arachidonic Acid	10 ul	1 mmol/l
Collagen	10 ul	2 ug/ml

The Payton dual channel aggregation module was allowed to warm up for 20 minutes. Specific glass tubes and magnetic stirrers for the aggregometer were siliconised with Repelcote for 30 minutes and rinsed 5 times with distilled water and dried.

The Chart paper was set at 20 mm/min and the sensitivity at 50 mV for both channels. The temperature was set at 37°C and the stirbar at 11 r.p.m. Channel 1 was set at 0.5 cm to 5.5 cm while Channel 2 was set at 4.5 cm to 9.5 cm

3.3.2 Phase Contrast Time Sequence Light Microscopy

Platelets were obtained from three healthy volunteers using the same method as described under platelet aggregation. One millilitre of the plasma containing the adjusted platelet count was placed on a glass slide and a cover was placed over the plasma. One hundred microlitres of S.T.D. was added to the side of the cover slip so that it would diffuse under the cover slip. Photographs were taken at 30 second intervals at the centre of the slide. The photographs were taken using a Reichert Univar Microscope. A magnification of 400 times was used with a phase contrast system. The shutter speed was 1/30 second. 64 ASA daylight film was used.
A field with aggregates containing more than 20 platelets was considered a positive response (Heyns et al, 1979).

3.4 RESULTS

3.4.1 Platelet Aggregation

Examples of the platelet aggregation are shown in figures 3.1 - 3.10. These were all duplicated. There was a normal response to ADP, adrenaline, ristocetin, and collagen. The ristocetin and ADP showed single waves without lag phases. The adrenaline showed a primary first wave of aggregation in the first 2 waves. A second wave occurred in the next 4 minutes with 100% light transmission. The collagen showed a lag phase for 1 minute, followed by a single wave of aggregation in the next 2 minutes with 95% aggregation.

3.4.1.1 Sodium Tetradecyl Sulphate

Addition of less than 25 ul of S.T.D. failed to elicit a response. However, the 25 ul S.T.D. (figure 3.5) showed a primary wave response over 2 minutes with 50% light transmission. A secondary wave response occurred which was completed after a further 4 minutes and 40 seconds with 90% light transmission. Thirty five microlitres of S.T.D. (figure 3.6) caused a single wave of aggregation without a lag phase over a 1 minute 45 second time interval. Addition of 50 ul caused an almost identical reaction (figure 3.7), however there was now evidence of disaggregation.

This is shown by the tracing returning to the baseline. Addition of 100 ul of S.T.D. caused aggregation followed by rapid disaggregation (figure 3.8). Higher concentrations caused 100% light transmission (figures 3.9 and 3.10). These could be confused with platelet aggregation, however in these cases there was no visible platelet aggregate which is indicative of platelet lysis. In all the other cases (viz. figures 3.1 to 3.7) an aggregate was visible at the end of the experiment. The patients with liver cirrhosis platelet aggregation, in response to ADP and S.T.D., were the same as the healthy volunteers.

Platelet aggregation is regarded as essentially a qualitative test rather than a quantitative test. A response of more than 50% was defined as an adequate response. As platelet responses to S.T.D. in both the healthy volunteers and the patients with liver cirrhosis were greater than 50% in all cases, quantitative statistical analysis was deemed unnecessary (Heyns, personal communication).

3.4.2 Phase Contrast Light Microscopy

Initially normal platelets were visualised (figure 3.11). As the concentration of the sclerosant increased, the platelets aggregated and this is depicted in figure 3.12. However, when the amount of sclerosant at the site exceeded a certain level all the platelets were lysed (figure 3.13). These tests were repeated once for every healthy volunteer. The identical results were obtained.

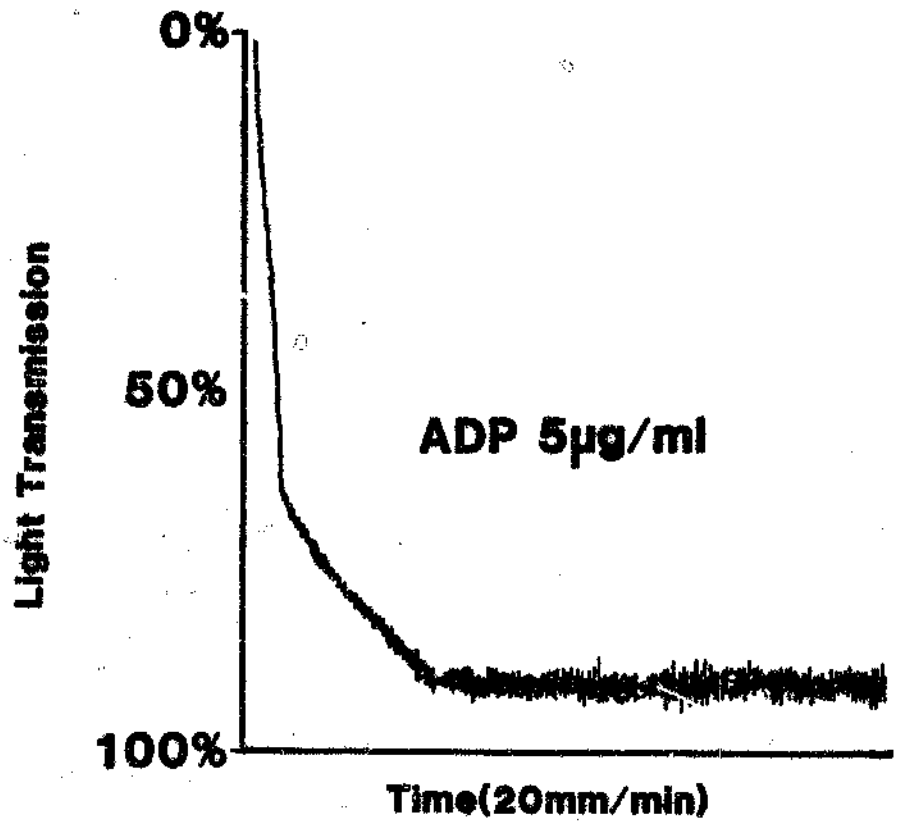


Figure 3.1
A tracing of a typical normal platelet aggregation response to addition of the agonist ADP.

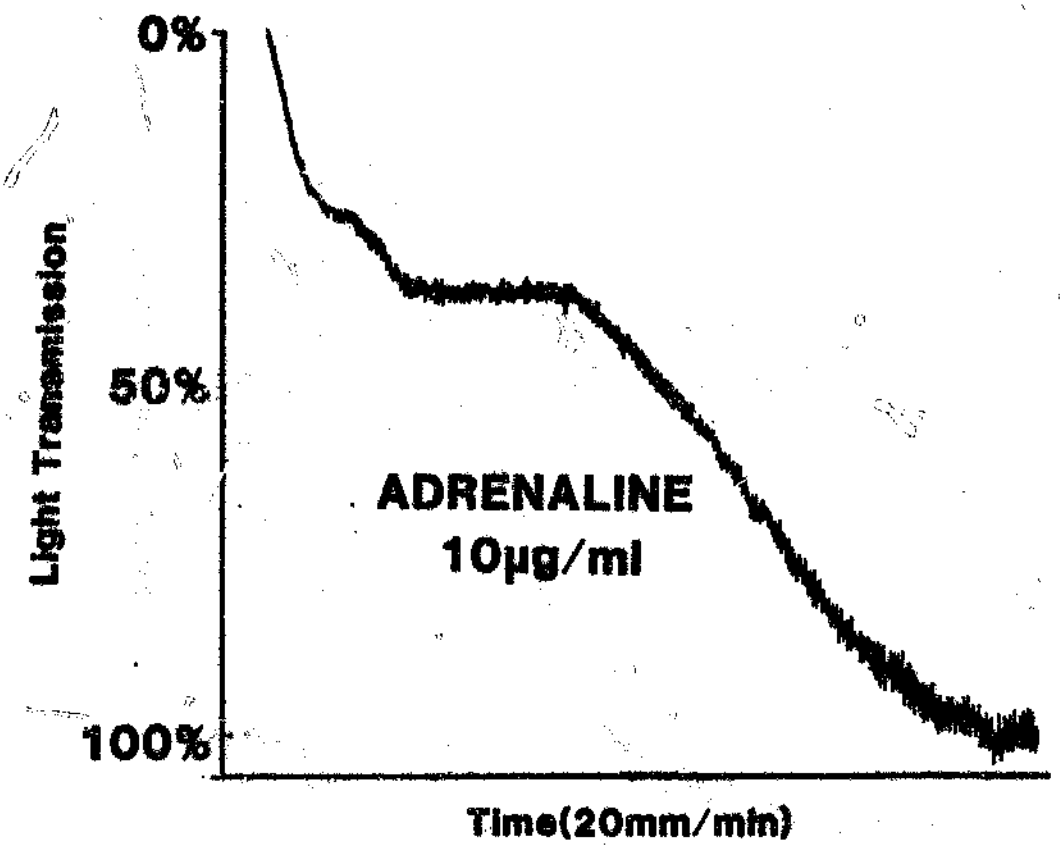


Figure 3.2
A tracing of a normal platelet aggregation as a result of addition of Adrenaline.

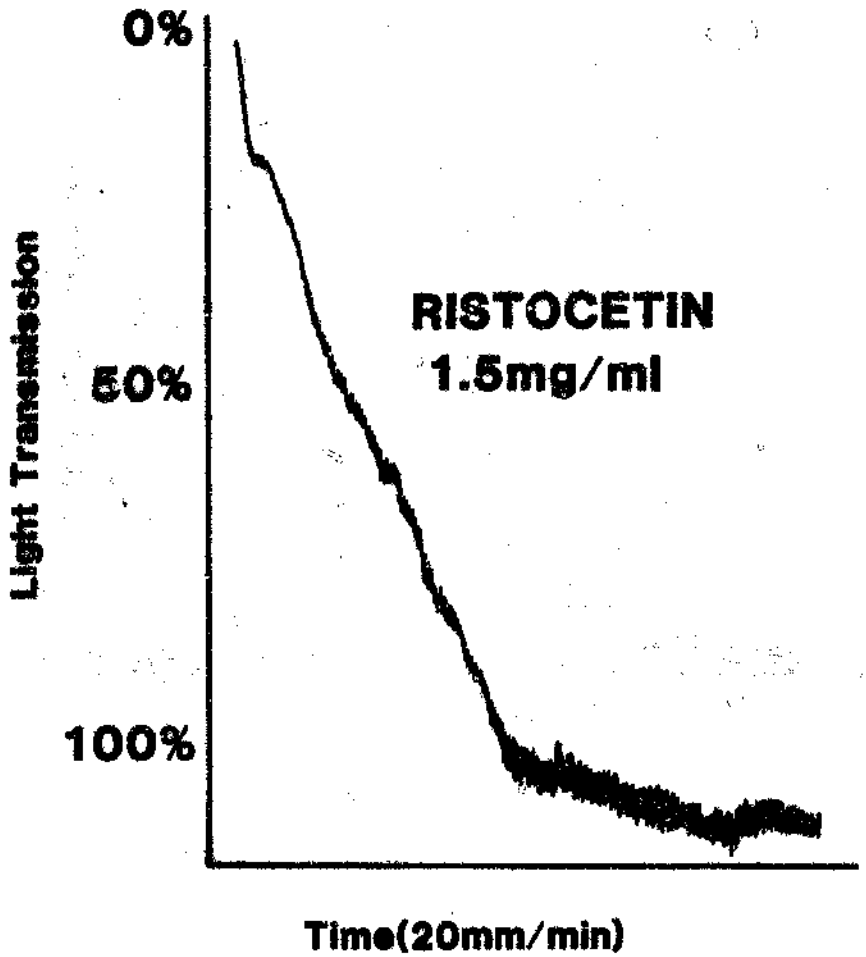


Figure 3.3
A platelet aggregation tracing showing the normal response to addition of ristocetin.

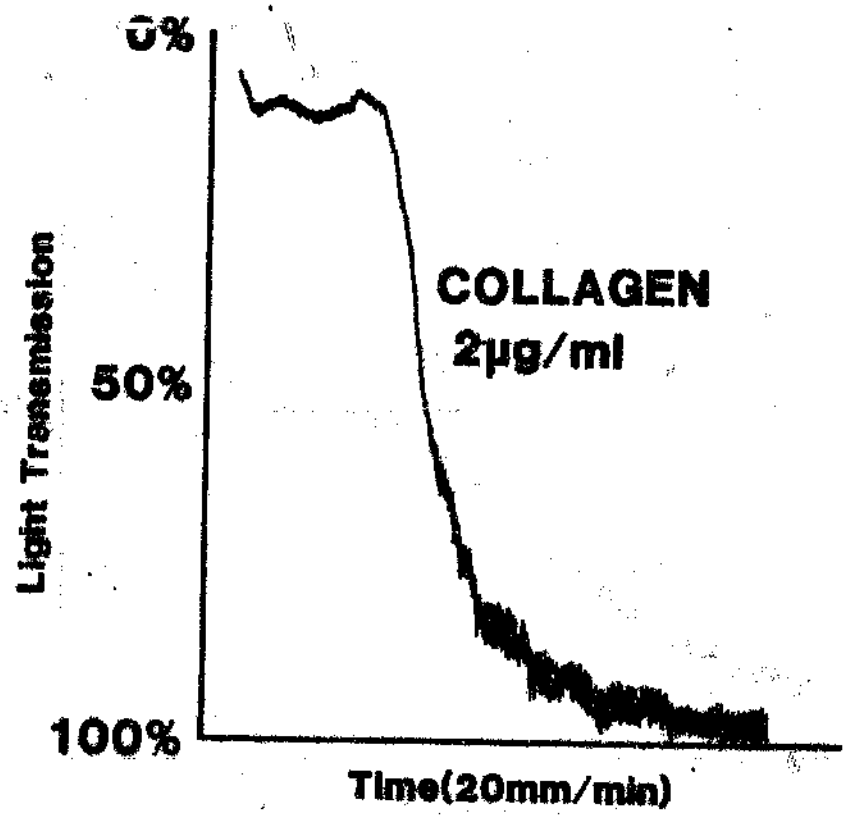


Figure 3.4
A normal platelet aggregation tracing following addition of collagen.

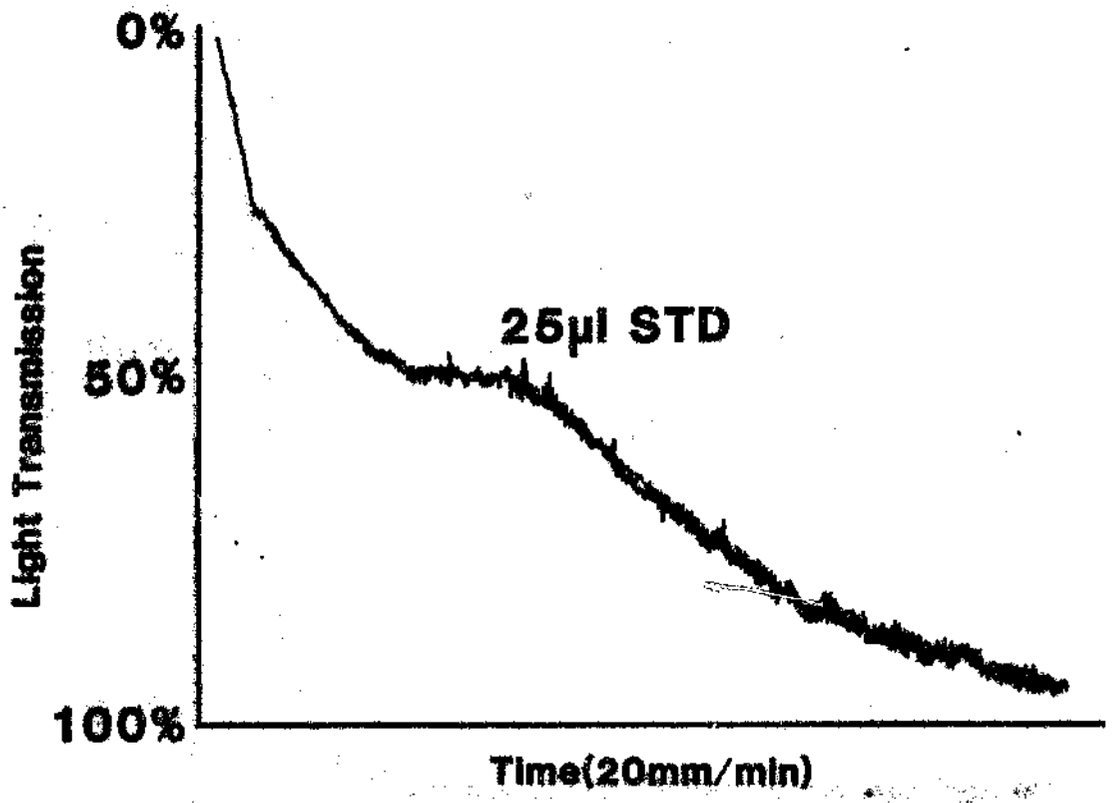


Figure 3.5
A tracing of a platelet aggregation occuring following addition of 25 µl of S.T.D.

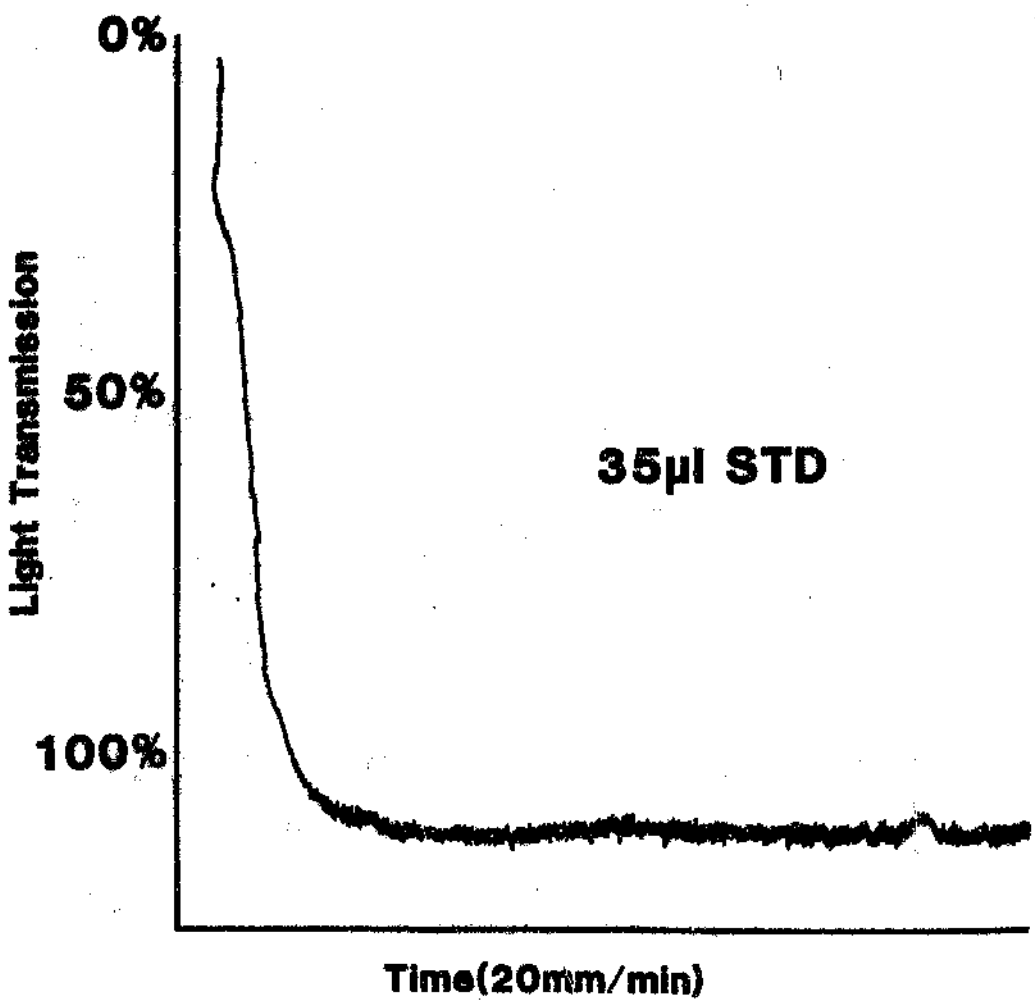


Figure 3.6
The platelet aggregation tracing which occured after addition of 35 µl of S.T.D.

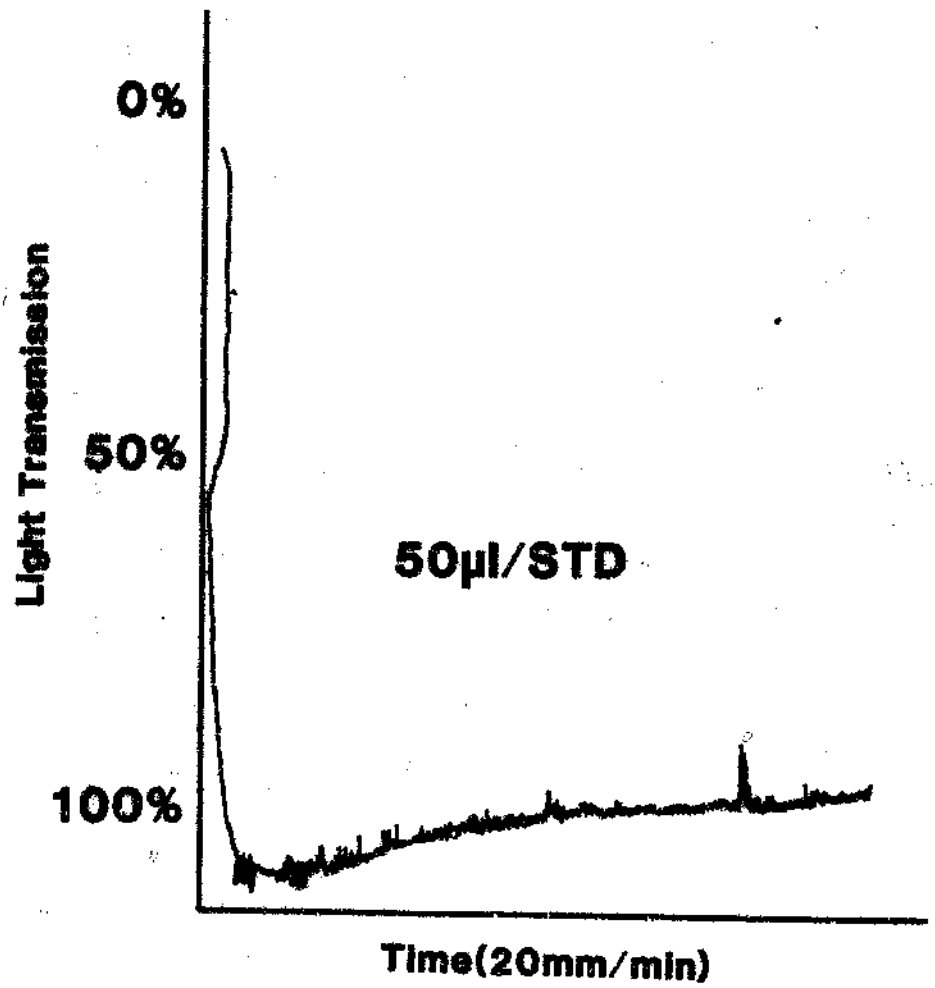


Figure 3.7
The platelet aggregation tracing after addition of 50 µl of S.T.D.

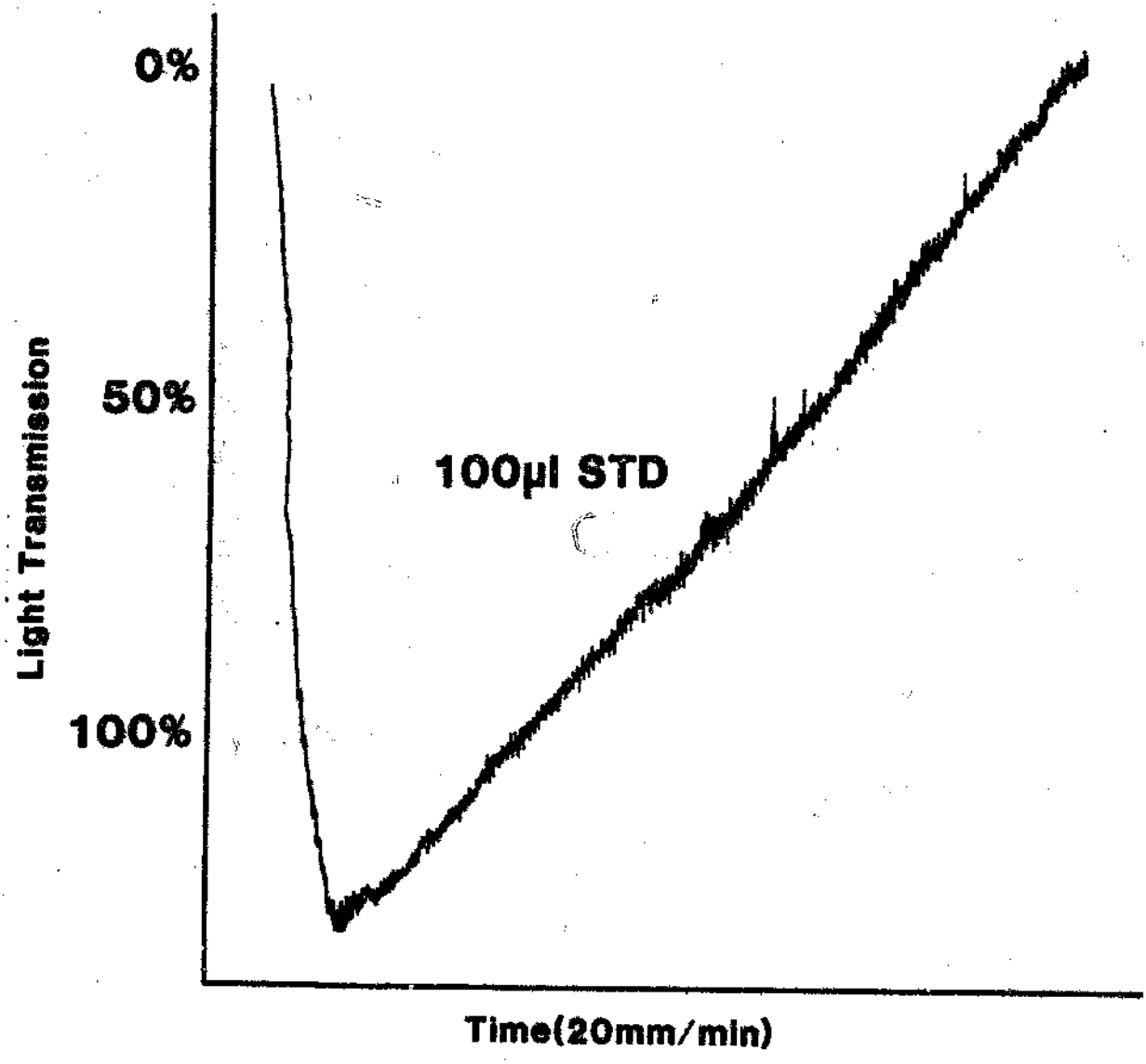


Figure 3.8
The platelet aggregation tracing following addition of 100 µl of S.T.D.

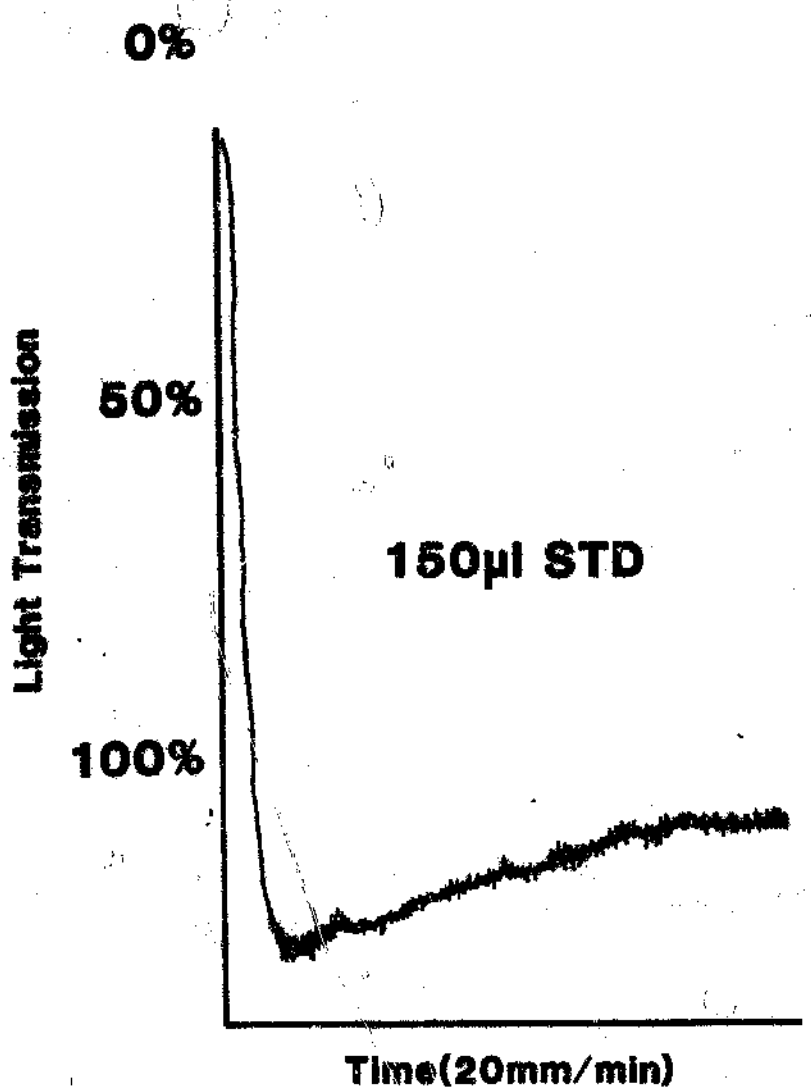


Figure 3.9
The platelet aggregation tracing after addition of 150 µl of S.T.D.

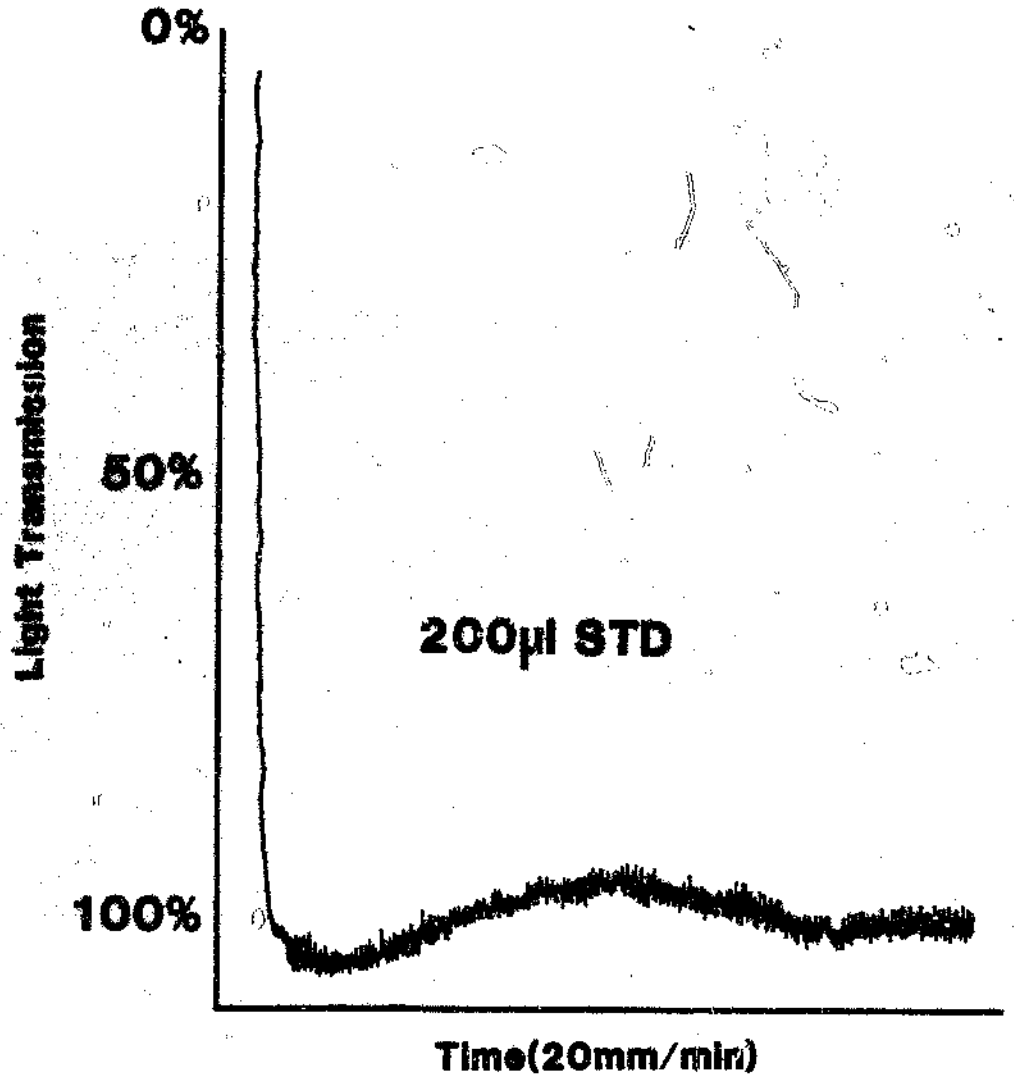


Figure 3.10
The platelet aggregation tracing after addition of 200 µl of S.T.D.

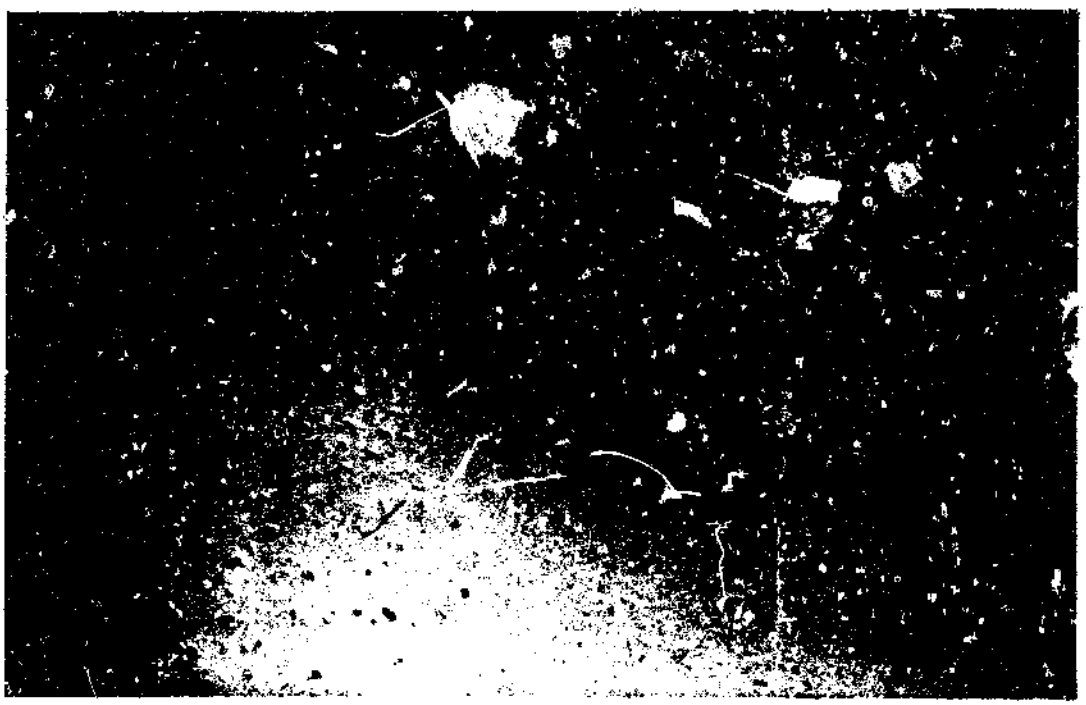


FIGURE 3.11 : Phase contrast microscopy of platelet rich plasma showing normal platelets.

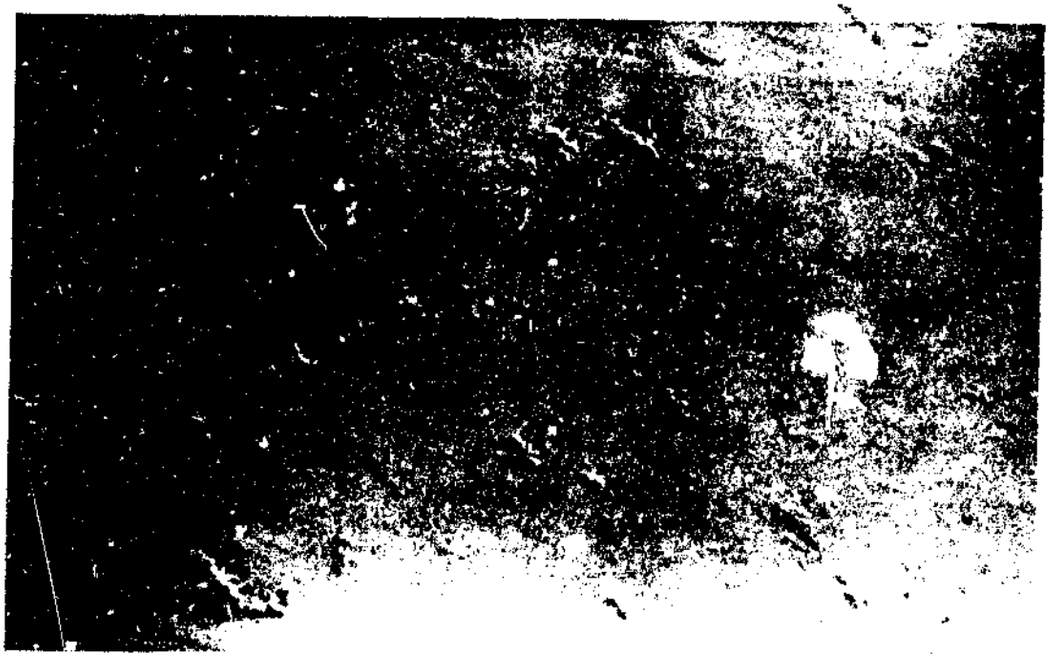


FIGURE 3.12 : Phase contrast microscopy at 30 seconds after addition of S.T.D. Note the visible aggregation.

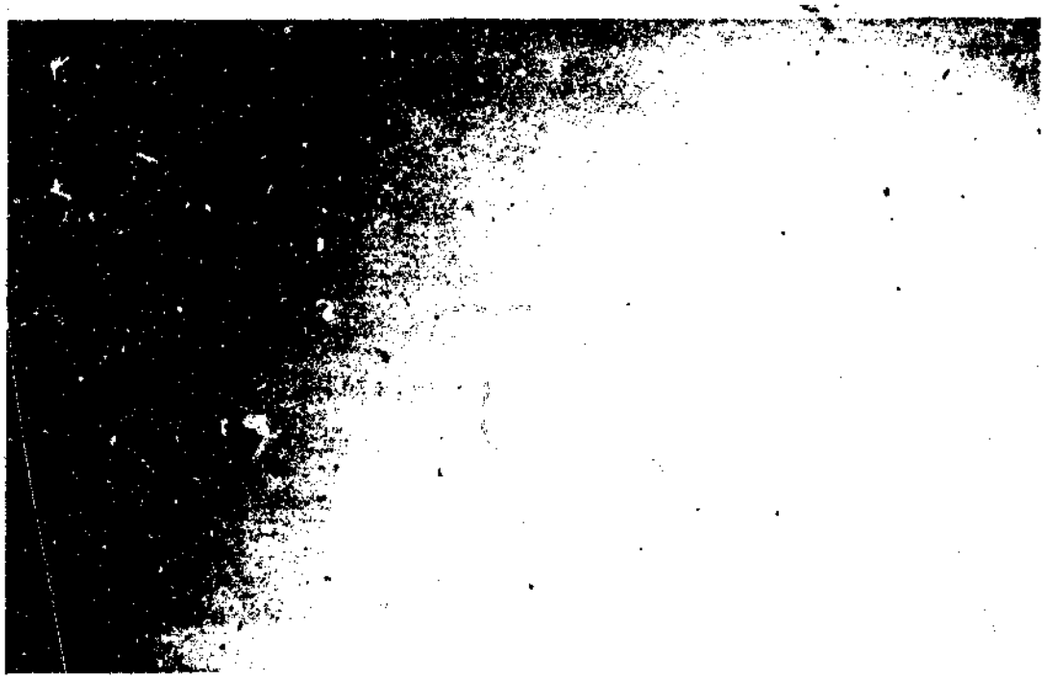


FIGURE 3.13 : Phase contrast microscopy two minutes post addition of S.T.D. Complete platelet lysis has occurred.

3.5 DISCUSSION

As previously discussed, platelets play an essential role in haemostasis. The first event that occurs with a breach in the vascular system is the formation of a cellular plug which is made up entirely of platelets (Zucker 1949). These platelets maintain their integrity and secrete their granular contents. Stripping of the endothelium and exposure of underlying collagen was demonstrated in the umbilical vein model (which is discussed in the chapter on endothelium) and platelets are known to adhere to collagen via a collagen receptor (platelet glycoprotein Ia) which participates with platelet glycoprotein IIa (Kunicki et al, 1988). This occurs at low shear rates, higher shear rates require von Willebrand factor (Baumgartner et al, 1980).

Platelet aggregation was assessed in vitro. In platelet aggregation the platelets stick specifically to each other. It requires prior platelet stimulation as well as active metabolism (Bennet et al, 1990). The agonists tested revealed normal aggregation patterns and the significance of these aggregations.

3.5.1 ADP (Figure 3.1)

ADP elicited a single wave of aggregation in both the healthy volunteers and the patients with liver cirrhosis. It is thought that ADP is one of the primary platelet activators in that it is released from damaged tissue and red blood cells (Gaarder et al, 1961).

The capacity to secrete these dense granules is cyclooxygenase dependent and is therefore inhibited by aspirin and anti-inflammatory drugs (Charo et al, 1977). ADP activation is postulated to be mediated via a receptor on the platelet surface although this has yet to be positively identified (Jefferson et al, 1988).

3.5.2 Adrenaline (Figure 3.2)

Adrenaline elicited a classical primary "first wave" aggregation, which is not associated with secretion, followed by a secondary wave where secretion occurs. The primary wave is reversible whereas the secondary wave is not. The mechanism of action of adrenaline is to bind to alpha-2 adrenergic receptors on the platelet (Newman et al, 1978). The receptor action is mediated via guanine nucleotide-binding protein, Gi, which inhibits adenylate cyclase (Kim et al, 1987).

3.5.3 Ristocetin (Figure 3.3)

Ristocetin aggregation showed a single wave without a lag phase. Ristocetin is an antibiotic. It was found to aggregate normal platelets but not to aggregate platelets of patients with von Willebrand's disease (Howard & Firkin, 1971). Coller (1983) has shown that positively charged ristocetin binds to platelets which alters the charge on the surface thus enabling vWF to bind to its receptor.

3.5.4 Collagen (Figure 3.4)

Collagen induced single wave aggregation after an initial lag phase. This is a classical response which collagen elicits. Collagen also elicits adhesion which was not tested for. The mechanism for adhesion differs in that it can be caused by both monomeric and fibrillar collagen (Brass et al, 1974). The reason for the lag phase is that after the fibrillar collagen binds to the platelet, the platelet synthesizes thromboxane and ADP which are released and act as agonists (Charo et al, 1977).

3.5.5 Sodium Tetradecyl Sulphate

Addition of extremely small volumes (i.e. 10 ul, 15 ul and 20 ul) failed to elicit a response. The response that 25 ul, 35 ul and 50 ul was elicited would appear to be of extreme importance. Although Stroncek et al,(1985) showed that sclerotherapy induced red blood lysis and the lysate has the capacity to induce platelet aggregation, it has not been shown previously that S.T.D. can induce platelets directly to aggregate. This finding is dose dependent. The mechanism by which it induces aggregation is unknown.

S.T.D. acts as an anionic detergent and is used to alter charges of proteins for electrophoresis. It is possible that S.T.D. would act in a similar way and directly alter the charge on the membrane of the platelet, thus facilitating aggregation. An

alternate explanation would be that S.T.D. damaged platelets and lyses them. They then release their contents which include agonists contained in their granules (which has been discussed earlier). The remaining platelets are thus able to form a single wave of aggregation. Should the concentration exceed a critical point, the S.T.D. interferes with subsequent aggregation and this would explain the disaggregation which occurs slowly when 50 ul of S.T.D. is added and rapidly when 100 ul of S.T.D. is added.

The higher volumes, i.e. 150 ul and 200 ul, caused an increase in optical density, which is the finding which occurs post aggregation. However, no clot was visible and it would have appeared that the platelets had all been lysed. This finding was confirmed on the time phase contrast light microscopy which showed that aggregation did occur at a specific local concentration. However when the local concentration exceeded a specific point lysis occurred.

Setting aside the controversy as to platelet dysfunction in liver cirrhosis, in this study low dose S.T.D. was as effective in inducing platelet aggregation in platelets from cirrhotic patients as it was with normal platelets. Similarly patterns of platelet lysis induced by high dose S.T.D. were unaffected by the presence of cirrhosis.

3.6 CONCLUSION

In conclusion, S.T.D. has been shown to cause platelet aggregation at specific low doses. From a haematological point of view, this in itself might prove to be an important finding, as non physiological agonists such as ristocetin (which is discussed earlier), have in the past proved to be important tools in elucidating platelet function. In the clinical situation the aggregation would occur at a site which is distal to the injection, i.e. downstream, where dilution has occurred. At the site of injection initially platelets are destroyed. However, platelets would also be activated at the site of injection when it has diluted to a specific critical concentration.

CHAPTER 4

4. COAGULATION

4.1 INTRODUCTION

The in vitro effect of sclerosants on the coagulation pathway have not been fully elucidated (Sivak et al, 1990). In 1967, Fegan in relatively crude experiments, found that Sodium Tetradecyl Sulphate caused a hypocoagulable state. In 1985 Stroncek et al, confirmed that Sodium Tetradecyl Sulphate in high concentrations caused the activated partial thromboplastin time, the prothrombin time and the thrombin time of plasma to be prolonged beyond assay limit times. In 1987 Kang et al showed that the recalcification time of plasma is prolonged on addition of ethanolamine oleate, another sclerosant, and this prolongation was felt to be primarily due to ethanolamine. In vitro studies of the sclerosant in concentrations used clinically demonstrated that fibrinogen was directly damaged by the sclerosants (Jacobson et al, 1984). That study was aimed to evaluate the advisability of the practice of addition of thrombin to the solution prior to sclerotherapy. In view of the relative paucity of available information, the effects of Sodium Tetradecyl Sulphate on in vitro coagulation are evaluated in this thesis. A detailed review of coagulation is beyond the scope of this thesis. However, the following is a brief review of the coagulation pathway with particular emphasis on the functions of the factors studied.

4.2 COAGULATION OVERVIEW

4.2.1 The Evolution of the Coagulation System

Coagulation in the human is a complex network of interrelated chemical reactions. These reactions ensure that coagulation (i.e. transforming blood from a sol to a gel) occurs only at specific sites and also enables this process to be reversed when required. Molecular evolution would require that this complex system evolved from a simple system. The most common mechanism by which this occurs is duplication of genes whose "offspring" have divergent function. This would explain an increase in the number of steps in the coagulation pathway (Patty et al, 1990).

In the invertebrate horse shoe crab, coagulation has a dual role in that it immobilizes invading bacteria. Human coagulation probably evolved from a similar mechanism. The evidence for the above is the close homology that Prothrombin, Factor VII, IX and X share with each other and with haptoglobin and C_1r and C_1s, suggesting that a common ancestral protein which was part of a general defence system diverged after gene duplication and this led to specialization of the clotting mechanism (Brenner, 1988). An important event in the evolution of the coagulation system was the acquisition of a calcium binding domain which made the coagulation dependent on calcium ions and phospholipid surfaces. This enabled the

coagulation cascade to be localized to the site of endothelial damage (Mann et al, 1988).

4.2.2 The Coagulation Cascade

The classic cascade pathway is a two dimensional representation of current understanding of the coagulation pathway. The factors involved are not only involved in coagulation but also in fibrinolysis as well as in initiating the inflammatory response. The cascade has positive feedback as well as negative feedback control mechanisms.

The initiation of the intrinsic pathway is due to contact activation. This has particular relevance as it is likely that sclerotherapy would activate this area of the pathway.

4.2.2.1 Factor XII

Factor XII would appear to be the first protein involved in the cascade process. Landerman et al (1962) described the lack of "permeability factor" i.e. Factor XII as a cause of hereditary angioneurotic oedema. Factor XII can be activated by enzymatic cleavage of a protease or by a negatively charged surface. The major activator is thought to be the subendothelial vascular basement membrane, however collagen as well as a variety of the components of the basement membrane do not

initiate coagulation in normal plasma and the role of the basement membrane remains therefore controversial. The precise mechanism by which negatively charged surfaces cause Factor XII activation is unknown. Factor XII is activated by proteases which cleave it to produce smaller molecules which are active. The major inhibitors of Factor XII are C1 inhibitor (the first component of the complement cascade), antithrombin III, as well as tissue plasminogen activator inhibitor. One major substrate of Factor XIIa is prekallikrein (Fletcher Factor). Seventy-five percent of Fletcher Factor which circulates is bound to high molecular weight kininogen (HMWK) (Scott, 1980). Activation involves limited proteolysis and conversion of prekallikrein to kallikrein can be accomplished by either XIIa or XIIf (Hageman Factor Fragment) (Wuepper et al, 1972). The two major inhibitors of kallikrein are alpha-2 macroglobulin and C1 inhibitor (van der Graaf, 1983). It has been postulated by van der Graaf et al (1983) that HMWK protects kallikrein from C1 inhibitor as the functional site of C1 inhibitor has been localized to the light chain of kallikrein which is the same area where HMWK binds. Kallikrein activates Factor XII via a positive feedback loop. A diagram of this pathway is represented in Figure 4.1.

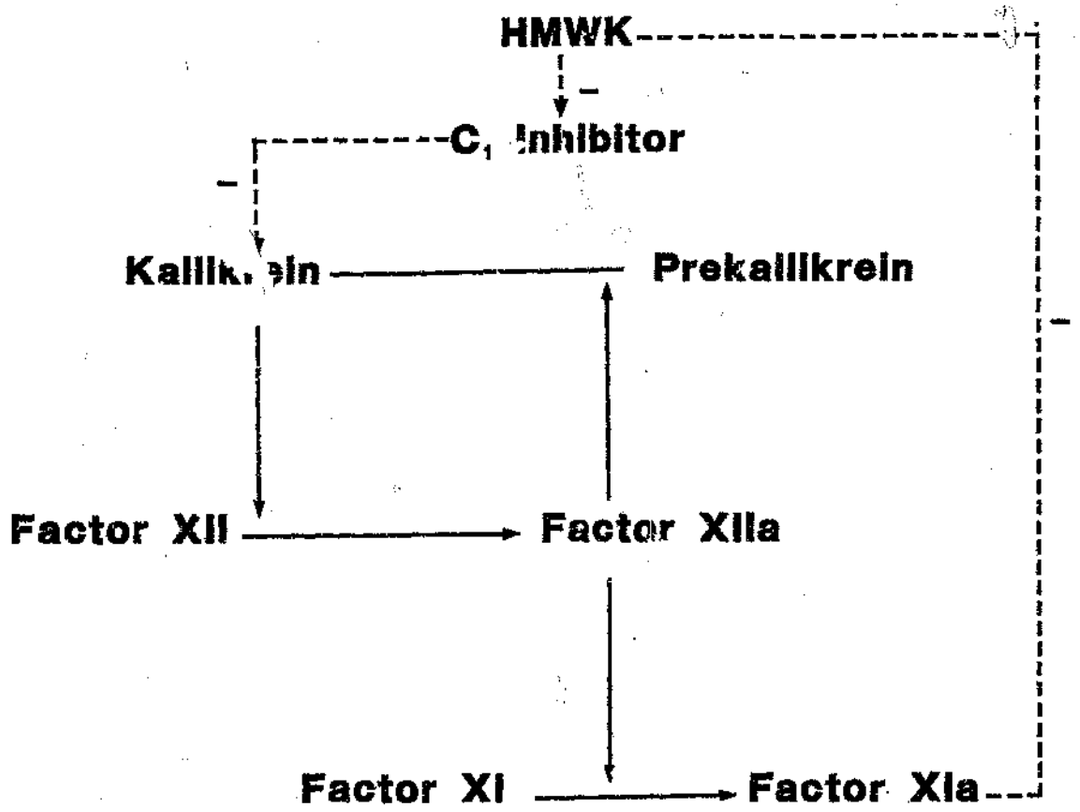


FIGURE 4.1 : A simplified diagram of the contact phase of the coagulation pathway. Note the positive feedback system of amplification of Factor XIIa which activates Prekallikrein. The negative feedback is shown where Factor XIa inactivates High Molecular Weight Kininogen which then no longer inhibits C_1 inhibitor. This allows C_1 inhibitor to inactivate Kallikrein and turn off the system.

A paradox of Factor XII has been to explain a lack of bleeding problems which occur clinically. Although Factor XII deficiency leads to a prolonged PTT, these patients tend to suffer from a thrombotic tendency rather than a bleeding problem. Ratnoff et al, (1968) have shown that these patients have an increased incidence of myocardial infarction, the basis of which is postulated to be the result of diminished fibrinolysis.

4.2.2.2 Factor XI

Another major substrate of Factor XIIa is Factor XI (Plasma Thromboplastin Antecedant). Factor XI is a gamma globulin two chain homodimer (Bouma, 1977). Factor XI consists of two almost identical molecules held together by disulphide bonds. Activation occurs after limited digestion by Factor XIIa (Bouma, 1977). It would appear that kallikrein is not necessary for the reaction but that HMWK is.

The only other activator of Factor XI is trypsin The major inhibitor of Factor XI is alpha-1 antitrypsin. The action of kallikrein on XI and XII is an example of amplification or positive feedback. Factor XIIa, which also results from cleavage of kallikrein allows more kallikrein to be activated. An example of

negative feedback is that Factor XI acts on HMWK which then is inactivated and this allows C1 inhibitor to inactivate kallikrein.

4.2.2.3 Factor IX

The action of Factor XIa is to activate Factor IX. Factor IX was first described by Biggs et al, (1932) who described a patient with haemophilia and normal Factor VIII levels and called the disease Christmas disease. It has subsequently also been called haemophilia B. Factor IX is a vitamin K-dependent factor, containing unique gamma glutamyl carboxyl acid residues at the N terminal end of the molecule which undergoes post-ribosomal modification by vitamin K so that calcium binding at the carboxyl group can occur. Factor IX is similar in structure to Factor X and Protein C, which is discussed later. Factor IX is a single chain glycoprotein containing approximately 20% carbohydrate. Its molecular weight is approximately 55,000. Due to its homology to the other Vitamin K dependent factors, Katayama et al (1979) have proposed that all vitamin K dependent factors have evolved from a common ancestor.

Factor XI activates Factor IX in the presence of calcium. This is achieved by cleavage of two internal peptide bonds. Factor IX can also be activated by Factor VII and tissue factor. This represents a bypass mechanism and its precise role in vivo has not yet been determined. Factor IX can be activated by Russell's viper venom. Factor IXa is inhibited by antithrombin which is greatly enhanced in the presence of heparin. It is also inhibited by hirudin which is present in the saliva of leeches (Hirsh, 1991). Factor IX is synthesized in the liver and, in the presence of activated Factor VIII, calcium, and phospholipid, activates Factor X.

4.2.2.4 Factor VIII

Factor VIII was first described by Addis in 1911. Factor VIIIc is separate from von Willebrand Factor (vWF) although they circulate in the plasma as a complex. Factor VIIIc is a product of an X-chromosome linked gene, while vWF is a product of a gene on chromosome 12.

Using SDS-page analysis, multiple bands of Factor VIII have been identified, the majority in the region of 79,000 to 80,000. The DNA sequence has been determined as well as the protein's 2351 amino acid sequence (Vehar,

1984). It is interesting that there is homology of the Factor VIIIa domain with Factor V as well as caeruloplasmin. Factor VIII's action is that of a true cofactor, as previously stated, and accelerates the cleavage of Factor X to Factor Xa caused by Factor IX. Factor VIIIc activity is lost after thrombin activation but the precise mechanism is not yet understood.

4.2.2.5 Factor X

Factor X is also a serine protease whose structure is very similar to Factor IX. Factor X has a molecular weight of 55,000. It also has gamma carboxyglutamic acid residues which can bind calcium ions after carboxylation by vitamin K. Activation of Factor X involves cleavage and loss of the so called activation peptide. Factor IXa alone can activate Factor X in the presence of phospholipid, albeit slowly. Factor VIII however cannot do this and is therefore a true cofactor (Hultin et al, 1978). Factor X is also activated by Russell's viper venom as well as trypsin.

4.2.2.6 Factor VII

Factor VII is synthesised in the liver. It is a single chain glycoprotein which requires Vitamin K for gamma carboxylation. It is

converted to its active form by tissue thromboplastin, thrombin, Factor XIIa and Xa. The activation is due to cleavage of a peptide bond which results in 2 chains forming, viz. a light and a heavy chain, which are joined by a disulphide bond. This factor shows close homology with the other Vitamin K dependent factors. Factor VII appears to activate small amounts of Factor X. Factor Xa has the ability to amplify the Factor VII. Factor X can therefore be activated by both the intrinsic and the extrinsic pathways.

4.2.2.7 Factor V

Factor V is a large polypeptide molecule. Immuno-electrophoresis shows that this protein is a gamma globulin (Greenquist et al, 1975). Thrombin increases Factor V activity which in turn accelerates the conversion of prothrombin to thrombin 278 000 times (Nesheim et al, 1979.) Protein C inhibits Factor V by cleaving it (discussed later). Factor V is found in platelets and is associated with the alpha granules (Ittyerah et al, 1981) and Factor Xa binding to platelets requires release of this Factor V or plasma Factor V. Factor V is also associated with endothelial cells and is thought to play the same role as it does with platelets. It is thought that injured

endothelial cells express Factor V on their surface.

4.2.2.8 Factor II

The next common step is the activation of Factor II (Prothrombin) to Factor IIa (Thrombin). As previously described Factor Va and Factor Xa as well as calcium are important and the phospholipid surface helps to localize the site of thrombin formation.

Prothrombin, with a molecular weight of 71 600, is synthesised in the liver and then secreted into the blood. Prothrombin is a glycoprotein which has gamma carboxyglutamic acid residues and is thus a vitamin K dependent factor. It also contains 2 regions which are called kringle structures. Carboxylation by vitamin K occurs on the lumenal side of the rough endoplasmic reticulum (Carlisle et al, 1980). Thrombin is formed when prothrombin is cleaved both at the internal arginine- threonine peptide bond which is after kringle 2, as well as at an arginine isoleucine bond located at the end of the light chain.

The natural substrates for thrombin are fibrinogen, which will be discussed in detail later, and Factor XIII. Thrombin has a positive feedback on Factors V and VIII as previously discussed and also activates Protein C.

4.2.2.9 Fibrinogen

The fibrinogen molecule is a dimer consisting of two sets of three pairs of polypeptide chains, viz. A alpha, B beta and gamma (Doolittle, 1984). Single copies of these genes are found on chromosome 4 and are closely linked, supporting the concept of a common ancestral origin which has duplicated and diverged. The principal proteolytic enzyme of fibrinogen is thrombin which hydrolyses arginine glycine bonds on the A alpha and B beta chains and releases fibrinopeptides A and B respectively (Hurlet-Jensen, et al, 1982). This exposes binding sites on the E domain of the fibrin monomer which bind to D domains on nearby gamma chains of different fibrin molecules producing the "DE staggered contact". D domains also bind to other D domains longitudinally, the so called DD long contacts and thus form fibrin polymers. A simplified diagram illustrating the above is shown in Figure 4.2

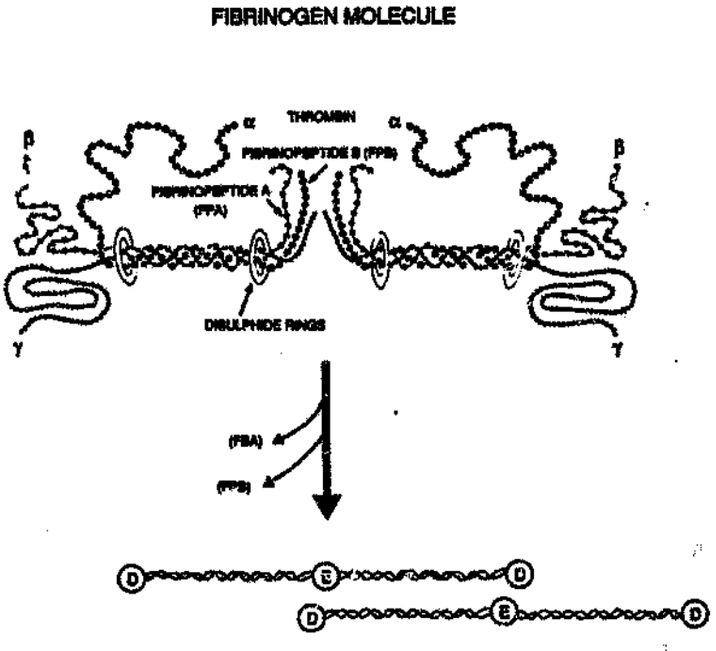


FIGURE 4.2 : A simplified schematic representation of the fibrinogen molecule and its conversion to fibrin.

Fibrin polymers are the final step in converting a liquid sol to a gel state. The fibrin polymers interconnect and form the lattice for the thrombin. Thrombin also converts Factor XIII from its inactive state to active Factor XIII. Factor XIIIa links the side chains of lysine and glutamine residues by isopeptide bonds and this strengthens the clot mechanically as well as making it more resistant to fibrinolysis.

4.2.2.10 Antithrombin III

The major inhibitor of thrombin is antithrombin III. Antithrombin is a protein with a molecular weight of 58,000. It contains an enzyme binding region as well as heparin binding sites. Prochownik et al, described the human gene for antithrombin in 1983. It is 16 kilobases in size. An interesting feature is that alternate splicing of primary transcripts may take place at two sites within the first intron. The result is either native antithrombin or a truncated product which remains within the cell. The function of the latter remains unknown. The mechanism of action of thrombin inhibition by antithrombin III is a mechanism by which complex formation occurs by means of an arginine serine interaction. (Rosenberg et al, 1973).

This reaction is greatly accelerated by the presence of heparin. The above authors also demonstrated that antithrombin inhibits Factors IXa, Xa, XIa and XIIa. Thrombin is also inhibited by heparin cofactor II. This protein is more specific for thrombin inhibition and also requires heparin as a cofactor.

4.2.2.11 Heparin

Heparin is a glycosaminoglycan with a molecular weight which varies from 3,000 to 100,000. Synthesis involves formation of carbohydrate protein connecting sequence followed by polymer chain assembly of the mucopolysaccharide. Heparin consists of alternating residues of uronic acid and glucosamine. The mechanism of action would appear to be that Domain 1 of the heparin molecule binds to a discrete region of antithrombin III which results in a conformational change. This accelerates factor Xa inhibition. Domains 1 and 2 of the heparin molecule bind to separate areas of the antithrombin molecule which induces a different conformational change which inhibits thrombin, factor IXa and factor XIa. Domain 3 of the heparin molecule aids in thrombin inhibition. Heparin also has multiple other roles and these include a role in angiogenesis, lipoprotein lipase activity and alteration in platelet aggregation (O'Brien et al, 1969).

4.2.2.12 Protein C (Owen, 1987)

This protein is a vitamin K dependent zymogen, activated by thrombin, which removes a tetradecapeptide. Its action is extremely important as unlike the other vitamin K dependent factors its mechanism of action is inhibition of coagulation, rather than activation of coagulation. Several families with protein C deficiency who have recurrent thrombotic episodes have been identified. The control mechanisms of protein activation appear complex. While free thrombin can activate Protein C, this activation is greatly enhanced by thrombin first binding to thrombomodulin. The latter is a protein produced by endothelial cells. Therefore intact functional endothelium (which would exist in vivo downstream from the site of injury) binds the thrombin which has been produced at the site of injury. This then activates Protein C which inactivates Factor V and Factor VIII. The cofactors for this reaction are thrombomodulin and Protein S. Protein S is a vitamin K dependent factor. Protein C also activates plasminogen activator which then initiates fibrinolysis.

4.2.2.13 Coagulation factor abnormalities in chronic liver disease.

Declining hepatic function due to chronic liver disease is associated with a decrease in plasma levels of all the factors of the coagulation pathway, with the exception of factor VIII. The basis of the observed deficiency is is due mainly to decreased synthesis (Joist, 1987). The vitamin K dependant factor reduction normally correlates with the extent of the hepatic disease. Factor IX, however, is usually only mildly reduced (Lechner et al, 1979). Factor V is often markedly reduced and is considered to be a more accurate predictor of hepatocellular damage (Joist, 1987).

Factor VIII levels are usually elevated in patients with chronic liver disease (Maisonneuve et al, 1977). Fibrinogen levels vary from normal to elevated or they may be decreased (Lechner et al, 1977). Dysfibrinogenemia is common in patients with liver cirrhosis (Soria et al, 1980), and is usually the basis for the explanation of the prolongation in the thrombin time which is frequently observed in patients with relatively normal fibrinogen levels. Disseminated intravascular coagulation, particularly the low grade forms, is often also associated with liver cirrhosis (Verstraete et al, 1974).

4.3 METHODOLOGY

All tests were performed on seven healthy volunteers.

4.3.1 Protein C determinations

The principle of the Protein C assay is that the Protein C in the sample is activated by a specific component of the snake venom Agkistrodon contortrix. Simultaneously the intrinsic system is activated by adding a cephalin surface activator and Protein C deficient plasma which contains all the other clotting factors in excess. The prolongation of the activated partial thromboplastin time will increase proportionately to the amount of activated Protein C as the latter inactivates Factor Va and Factor VIIIa.

Protein C was determined with the Behring kit, using the following technique:

The plasma was separated by mixing 1 part Trisodium citrate solution with 9 parts venous blood. It was centrifuged for 10 minutes at 1 700 g and the supernatant was stored at -20°C if the test was not done immediately. Samples that had been frozen were thawed at 37°C for 10 minutes and assayed within 2 hours. The patient's samples were prediluted with 1 part patient's sample to 9 parts of Owrens Buffer (pH 7.35). These predilutions were used within 10 minutes. The assay procedure was performed by pipetting the following reagents into a

test tube, which had been pre-warmed to 37°C ; 0.1 ml of the 1:10 dilution of the patient's sample, 0.1 ml Protein C deficient plasma (which consists of citrated human plasma with a Protein C concentration of less than 1%), 0.1 ml Protein C Activator (which is extracted from the venom of the Agkistrodon contortrix), 0.1 ml Neothromtin (which is a PTT reagent consisting of ellagic acid and vegetable phospholipids)

The above were all mixed and incubated for 4 minutes at 37°C in a waterbath. The order of pipetting was kept constant. 0.1 ml of Calcium chloride solution at 37 C was then added. A stop watch was used to determine the coagulation time.

A reference curve was drawn up on log/log graph paper and used to read off the results, as a percentage of the normal. Samples having results outside the range of the reference curve were retested using different predilutions. The normal reference range was considered to be 70 - 140%.

4.3.2 Factor V Assay

The principle of all the factor assays is that the sample is added to plasma which is deficient in the specific factor which is being assayed. The amount of factor present in the sample will proportionately shorten either the partial thromboplastin time or the

prothrombin time. The specific factor deficient plasma contains all the remaining coagulation factors.

Factor V activity was assayed using the following methodology:

Dilutions of normal and the patient's plasma were prepared. 1:5 was made up using 0.1 ml plasma and 0.4 ml buffer; 1:10 using 0.1 ml plasma and 0.9 ml buffer; 1:50 using 0.1 ml of a 1:10 dilution of plasma and 0.4 ml buffer; 1:100 using 0.1 ml of a 1:10 dilution of plasma and 0.9 ml buffer. The buffer used was Owrens Buffer (pH 7.35). Only one set of dilutions was made at a time and they were not allowed to stand longer than 30 minutes, as Factor V is very labile.

The following were pipetted in sequence into a clotting tube:

0.1 ml of Factor V deficient plasma; 0.1 ml dilution of normal/patient's plasma; 0.1 ml phenolised brain; 0.1 ml calcium chloride (0.025M). A stopwatch was started and the times were recorded. The times obtained were plotted on log/log graph paper using the following parameters: 1:5 = 200%; 1:10 = 100%; 1:20 = 50%; 1:50 = 20%; 1:100 = 10%. The results were always extrapolated from the 100% line and were determined as a percentage of normal.

4.3.3 Factor VII Assay

Factor VII assay was identical to the Factor V assay, however Factor VII deficient plasma is used instead of Factor V deficient plasma.

4.3.4 Factor VIII Assay

The dilutions of normal and patient's plasma in buffer were made up exactly the same as in the Factor V assay. The following were pipetted in sequence into a clotting tube:

0,1 ml Factor VIII deficient plasma; 0.1 ml dilution; 0.1 ml aPTT reagent containing cephalin. These were incubated at 37 C for 3 minutes. 0.1 ml calcium chloride (0.025M) was then added and a stopwatch was started and the clotting times recorded. The results were graphed on log/log paper.

4.3.5 Factor IX assay

Factor IX assay was done in the identical way to the Factor VIII, using IX deficient plasma.

4.3.6 Factor XI assay

Factor XI assay was done in the identical way to the Factor VIII, using Factor XI deficient plasma. However the incubation time was increased from 3 to 10 minutes because Factor XI is a contact activator.

4.3.7 Factor XII assay

Factor XII assay was done in the identical way to the Factor XI, using Factor XII deficient plasma.

4.3.8 Factor X Assay

Dilutions of normal and patient's plasma were prepared in the same way as the Factor V assay except that Factor V deficient plasma was substituted with Factor X deficient plasma.

4.3.9 Fibrinogen Assay

Fibrinogen was assayed by the method according to Ellis and Stransky. 0.5 ml of plasma was added to 5.5 ml of barbitone buffer. Three millilitres was transferred to the test cuvette and the remainder was used as a blank. Fifty microlitres of calcium chloride/thrombin solution was added to the test cuvette and inverted gently 6 times. The optical density of the test was read against the blank at 470 nm after 20 minutes.

4.3.10 The Kaolin Activated Partial Thromboplastin Time

The Kaolin Activated Partial Thromboplastin Time measures the intrinsic pathway and the common pathway via contact activation. The test consists of adding the following reagents in the following sequence:

0.1 ml of normal or patient's plasma; 0.1 ml aPTT reagent containing cephalin (supplied by Lennon Diagnostics). The mixture was incubated at 37°C for 3 minutes. 0.1 ml calcium chloride (0.025M) was then added and the clotting time was recorded. These tests were all performed in duplicate. Normal range for our laboratory is 27 - 41 seconds.

4.3.11 <u>The Prothrombin Ratio/International Normalised Ratio</u>

The Prothrombin ratio measures the extrinsic and common pathway using tissue (brain) thromboplastin as the activator. The test consists of adding the following reagents in the following sequence:

0.1 ml or normal or patient's plasma; 0.1 ml phenolised human brain. The mixture was incubated at 37°C for 60 seconds. 0.1 ml calcium chloride (0.025M) was then added and the clotting time was recorded. These tests were performed in duplicate. The prothrombin ratio was then calculated as the ratio between the patient's time in seconds divided by the normal time in seconds raised to the power of the International Sensitivity Index (ISI). The normal range for our laboratory is less than 1.25.

The prothrombin time is measured in seconds and by dividing the result of the test plasma by the control plasma the ratio is obtained. A major problem has been that various thromboplastins have different

sensitivities. This has had serious clinical implications for patients on coumadin oral anticoagulant therapy as dosage is dependent on the prothrombin ratio. In order to standardize prothrombin ratio worldwide, reference thromboplastin from the WHO Reference Preparations was obtained. The International Sensitivity Index (ISI) was obtained by comparing the reference thromboplastin with the test thromboplastin using standard arithmetical techniques (Dacie, 1991). The International Normalised Ratio is obtained by raising the ratio of the sample tested to the power of the ISI.

4.3.12 Fibrin/Fibrinogen Degradation Products

This test was done using the Thrombo-Wellcotest Latex Suspension HA08 kit. The principle of the test is that antisera are raised to highly purified preparations of human fibrinogen fragments D and E. After solid phase absorption to remove antibodies to all other serum proteins, the specific antibody globulins are extracted and used to coat by adsorption a suspension of latex particles in a glycine saline buffer. The sensitivity of the latex reagent is adjusted so that in the presence of FDP concentrations of 2 ug/ml or greater the latex particles clump together giving macroscopic agglutination. The test was performed in the following manner:

All samples were taken into bottles containing an inhibitor of proteolytic enzyme activity (soya bean

trypsin inhibitor) and treated with thrombin to remove unclotted fibrinogen. The sample was freed from cells and fibrin before preparing test dilutions. Two small glass tubes were marked 1 and 2. Two rings on the slide were similarly marked. 1:5 and 1:20 dilutions of the serum sample were prepared using glycine saline buffer pH 8.2 in tubes 1 and 2 respectively. Using the same pipette for both dilutions, 1 drop from test tube 2 was transferred to position 2 on the slide and similarly 1 drop fr test tube 1 to position 1. The latex suspension was thoroughly mixed by rapidly inverting the bottle 3 times, and 1 drop of the suspension was then added to each of the 2 positions. The mixtures were then stirred in turn and the slide was rocked to and fro for 2 minutes while looking for macroscopic agglutination.

The slides were interpreted as positive if agglutination took place. A positive result in position 1 indicated that FDP's were present in the original serum at a concentration in excess of 10 ug/ml while agglutination in position 2 indicated the original concentration to have been greater than 40 ug/ml.

4.3.13 Statistics

Statistical analysis was performed by the Institute for Biostatistics of the South African Medical Research Council. The analysis was performed with the appropriate analysis of variance at the 0.05 level of significance with the help of Tukey's Studentized Range (Keppel et al, 1980).

4.3.14 Quality Control

The laboratory where the tests were performed is a reference laboratory for coagulation studies. Prothrombin times and activated partial thromboplastin times were assayed in duplicate and the average times recorded. If there was a discrepancy of more than 4 seconds, the test was repeated. The reference assays were obtained from normal pooled plasma from 15 normal people. This normal pooled plasma was prepared daily. The standard reference curves for factor assays and Protein C were obtained from commercial lyophilised plasma which has a reference value for all coagulation factors. This was checked against normal pooled plasma. Reproducibility of all kits was assayed prior to using the kit. This was performed on normal plasma and assayed ten times. The coefficient of variation was less than 2.

Internal quality control is performed monthly in our laboratory and the laboratory also participates in international external quality controls, viz. the Quality Control of Australian Pathology and the World Health Organisation International External Quality Assessment Scheme in Blood Coagulation. The results of these international quality controls, during the period of this investigation, were consistently within the acceptable range.

4.4 RESULTS

After the plasma had been separated there was notable evidence of gross haemolysis which was dose dependent (Figure 4.3). The results of the levels of factors present after addition of different amounts of S.T.D. are all expressed as a percentage of the control.

4.4.1 Factor XII (Figure 4.4)

The Factor XII levels were not affected up to 150 ul of S.T.D. when the level dropped to 84%. Addition of 200 ul resulted in a further drop to 72% while 350 ul resulted in a reduction to 53%.

4.4.2 Factor XI (Figure 4.5)

The Factor XI levels did not significantly drop after addition of up to 100 ul of S.T.D. One hundred and fifty microlitres of S.T.D. caused a reduction to 88% and 200 ul to 80%. Three hundred and fifty microlitres caused a further reduction to 52%.

4.4.3 Factor X (Figure 4.6)

Factor X levels remained above 90% with addition of up to 50 ul. One hundred microlitres caused a reduction to 87%, 150 ul to 83% and 200 ul to 75%. Factor X levels were markedly reduced to 19% after addition of 350 ul of S.T.D.

4.4.4 Factor IX (Figure 4.7)

Factor IX levels were also all greater than 90% with addition of up to 100 ul of S.T.D. Further addition of S.T.D., viz. 150 ul, 200 ul and 350 ul, resulted in drops to 86%, 70% and 42% respectively.

4.4.5 Factor VIII (Figure 4.8)

Factor VIII levels remained at 90% or higher with up to 100 ul of S.T.D. One hundred and fifty microlitres of S.T.D. resulted in a drop to 74%, 100 ul of S.T.D. dropped the Factor VIII levels to 59% and 350 ul of S.T.D. dropped the Factor VIII levels to 43%.

4.4.6 Factor VII (Figure 4.9)

Factor VII levels showed a gradual decrease from 95% after addition of 10 ul of S.T.D., 94% after 25 ul of S.T.D., 92% after 35 ul of S.T.D., 90% after 50 ul of S.T.D., 85% after 100 ul of S.T.D., 80% after 150 ul of S.T.D., 67% after of 200 ul of S.T.D. and 29% after addition of 350 ul of S.T.D.

4.4.7 Factor V (Figure 4.10)

Factor V was resistant to addition of low doses of S.T.D. in that at up to 150 ul of S.T.D. the levels were greater than 90%. Two hundred microlitres of S.T.D. caused the level to drop to 53% and 350 ul caused a further drop to 22%.

4.4.8 Factor II (Figure 4.11)

Factor II levels remained greater than 90% with up to 100 ul of S.T.D. One hundred and fifty microlitres of S.T.D. resulted in a drop to 88%, 200 ul of S.T.D. caused a decrease to 74% and 350 ul to 38%.

4.4.9 Fibrinogen (Figure 4.12)

Fibrinogen levels remained greater than 90% with 25 ul of S.T.D. Thirty five microlitres caused a drop to 89%, at 50 ul it remained at 89% and at 100 ul it dropped to 84%. One hundred and fifty microlitres, 200 ul and 350 ul of S.T.D. resulted in a reduction to 79%, 64% and 38%.

4.4.10 Protein C (Figure 4.13)

Protein C proved to be the most sensitive indicator in that 10 ul of S.T.D. dropped the level to 90%. Twenty five microlitres of S.T.D. dropped the Protein C level to 73% of the control. This was statistically significantly lower than any of the other factors tested at this level of addition of S.T.D. ($p = 0.0001$). The level at 35 ul was 75% and at 50 ul of S.T.D. was 72%. The level at 100 ul of S.T.D. remained constant at 72%. One hundred and fifty microlitres of S.T.D. caused a reduction to 71% At 200 ul of S.T.D. the Protein C level fell to 61% and at 350 ul the level dropped to 30%.

4.4.11 Partial Thromboplastin Time (Figure 4.14)

The partial thromboplastin time remained essentially unchanged until the addition of 100 ul of S.T.D. Thereafter there was a marked increase in the time. Addition of 350 ul of S.T.D. resulted in a time of greater than 120 seconds.

4.4.12 International Normalised Ratio (INR) (Figure 4.15)

The INR remained below 1.20 on addition of up to 100 ul of S.T.D. One hundred and fifty microlitres of S.T.D. caused the INR to increase to 1.25, 200 ul to 1.63 and 300 ul resulted in the INR being prolonged to 6.6.

FIGURE 4.3 : A photograph illustrating haemolysis after addition of increasing volumes of S.T.D.

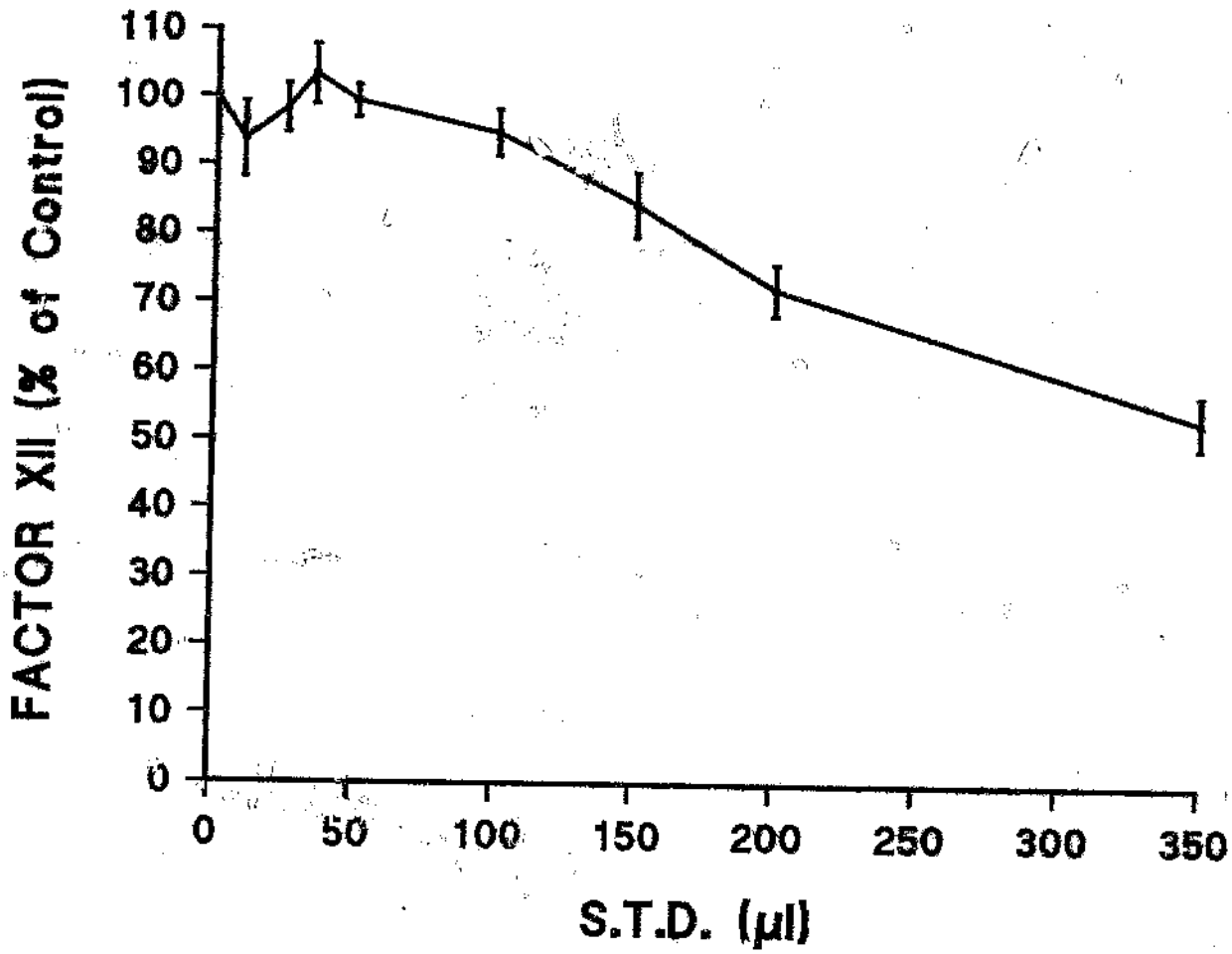


FIGURE 4.4

The in vitro effect of S.T.D. on Factor XII (Mean ± SEM)

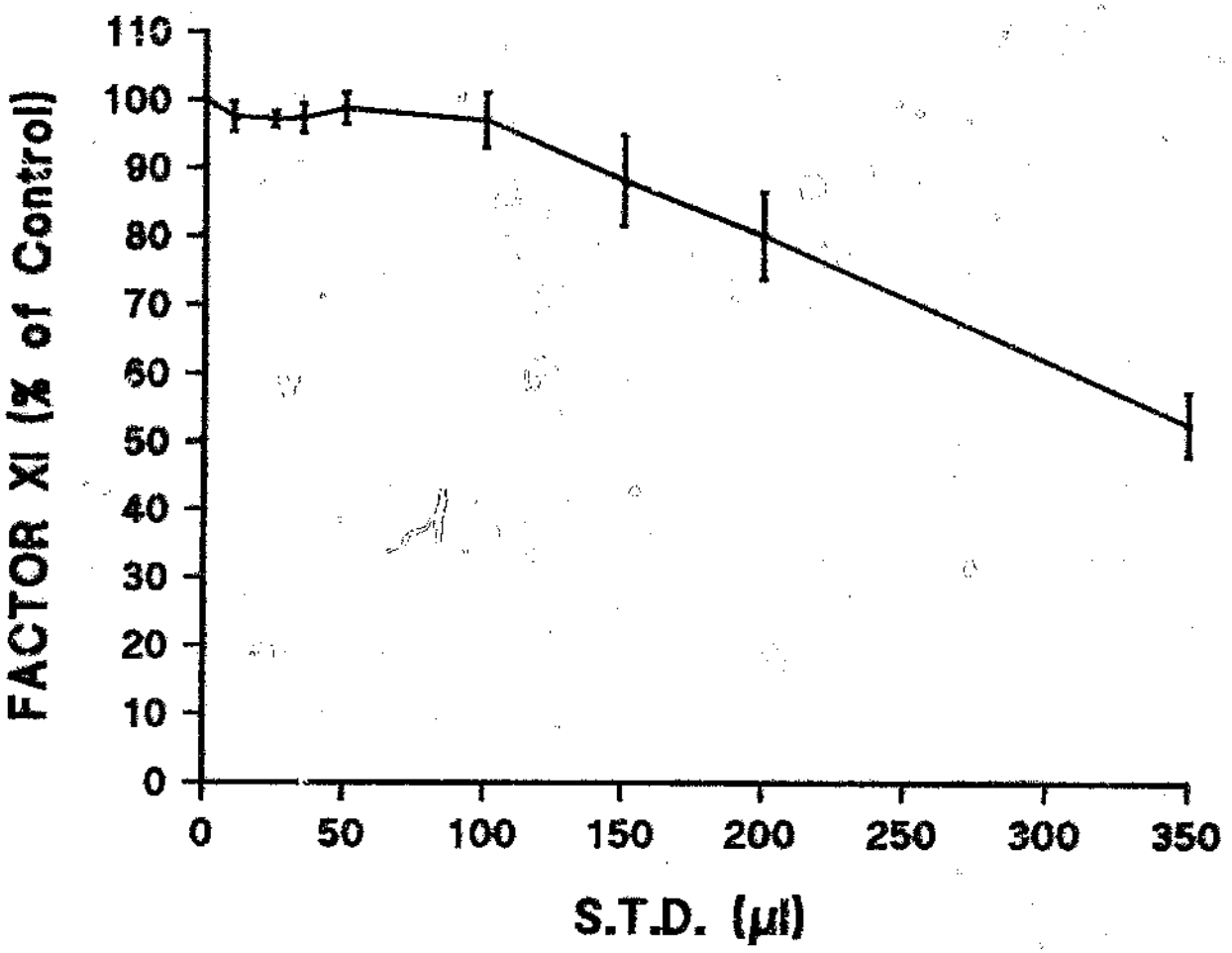


FIGURE 4.5

The in vitro effect of S.T.D. on Factor XI (Mean ± SEM)

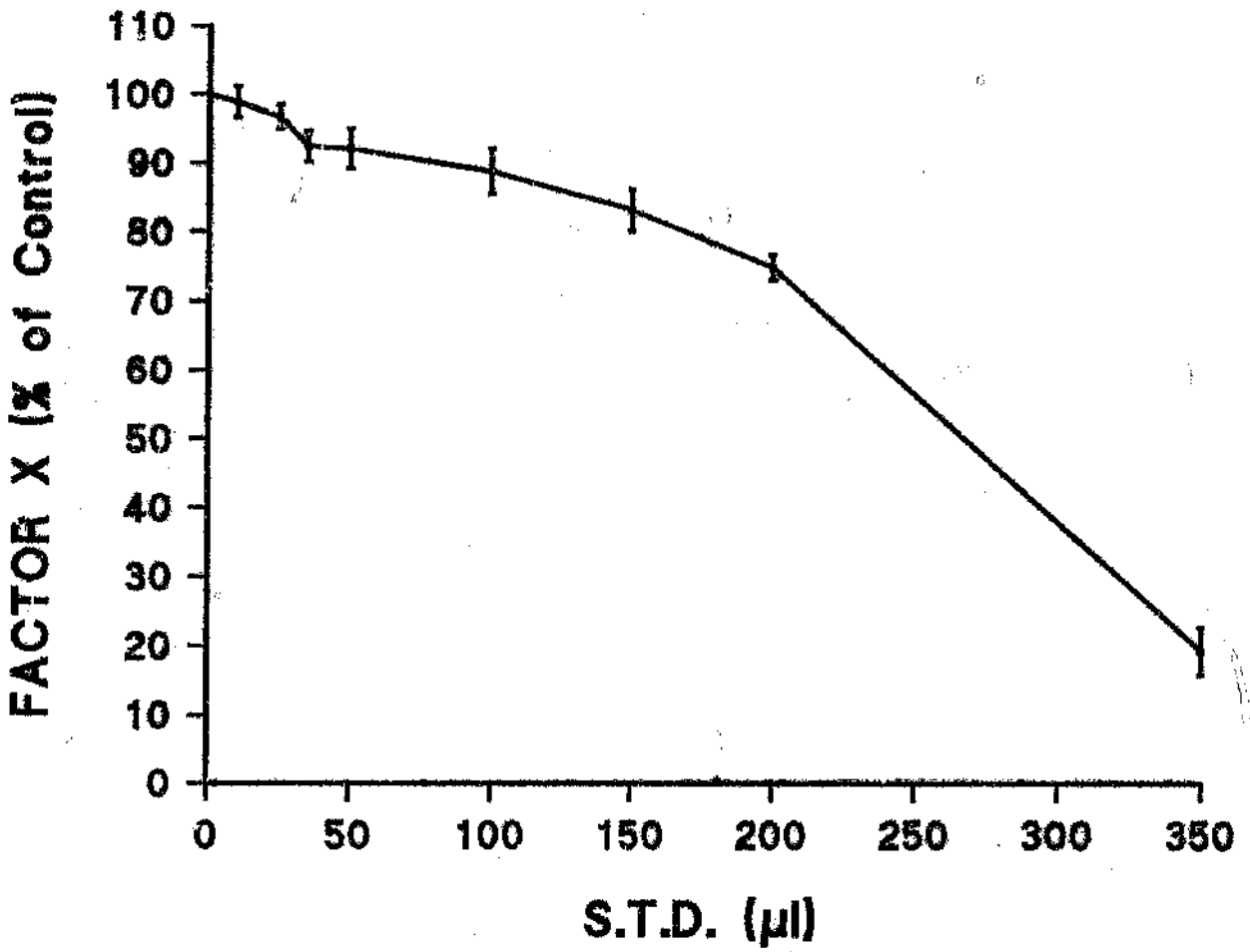


FIGURE 4.6

The in vitro effect of S.T.D. on Factor X

(Mean ± SEM)

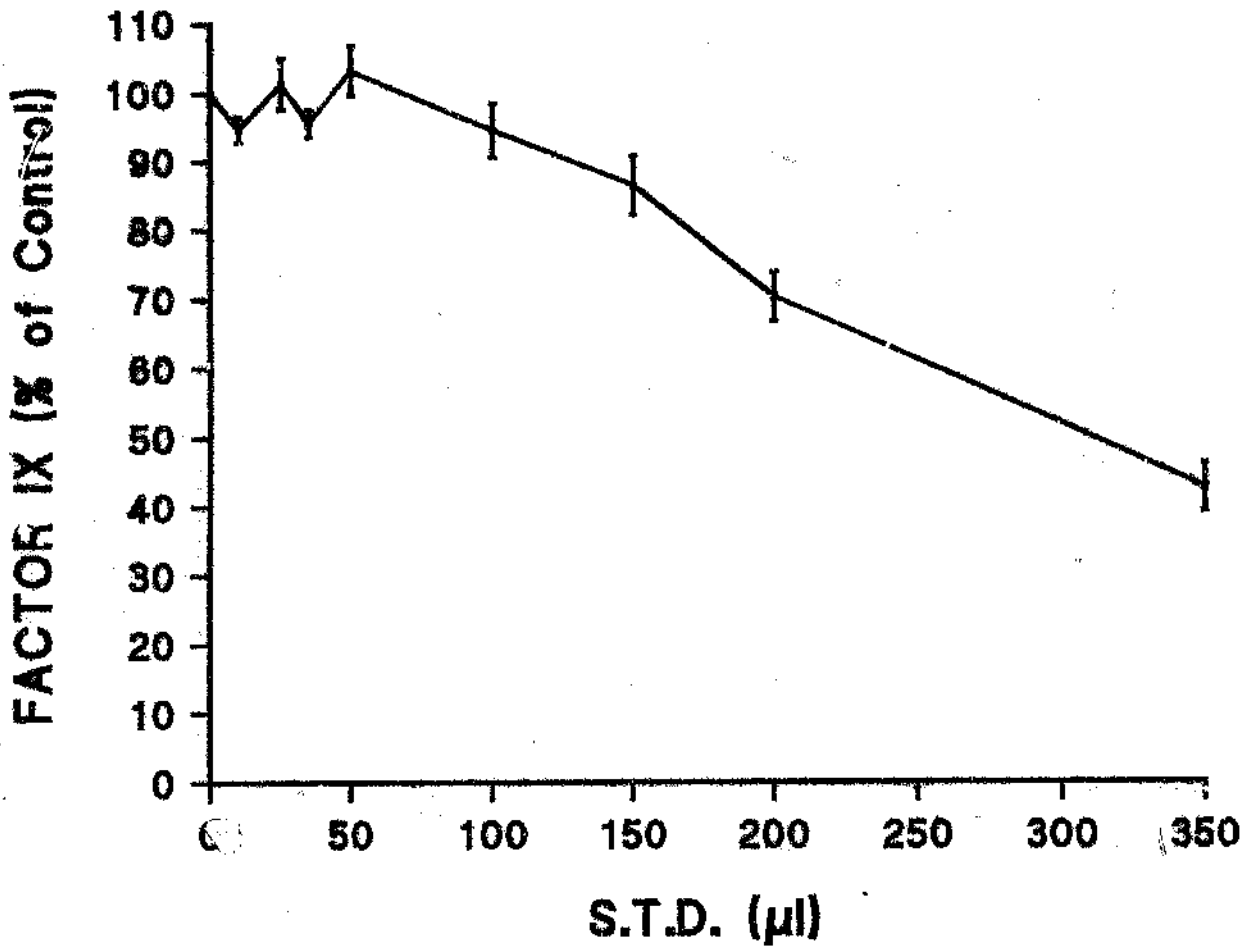


FIGURE 4.7

The in vitro effect of S.T.D. on Factor IX (Mean ± SEM)

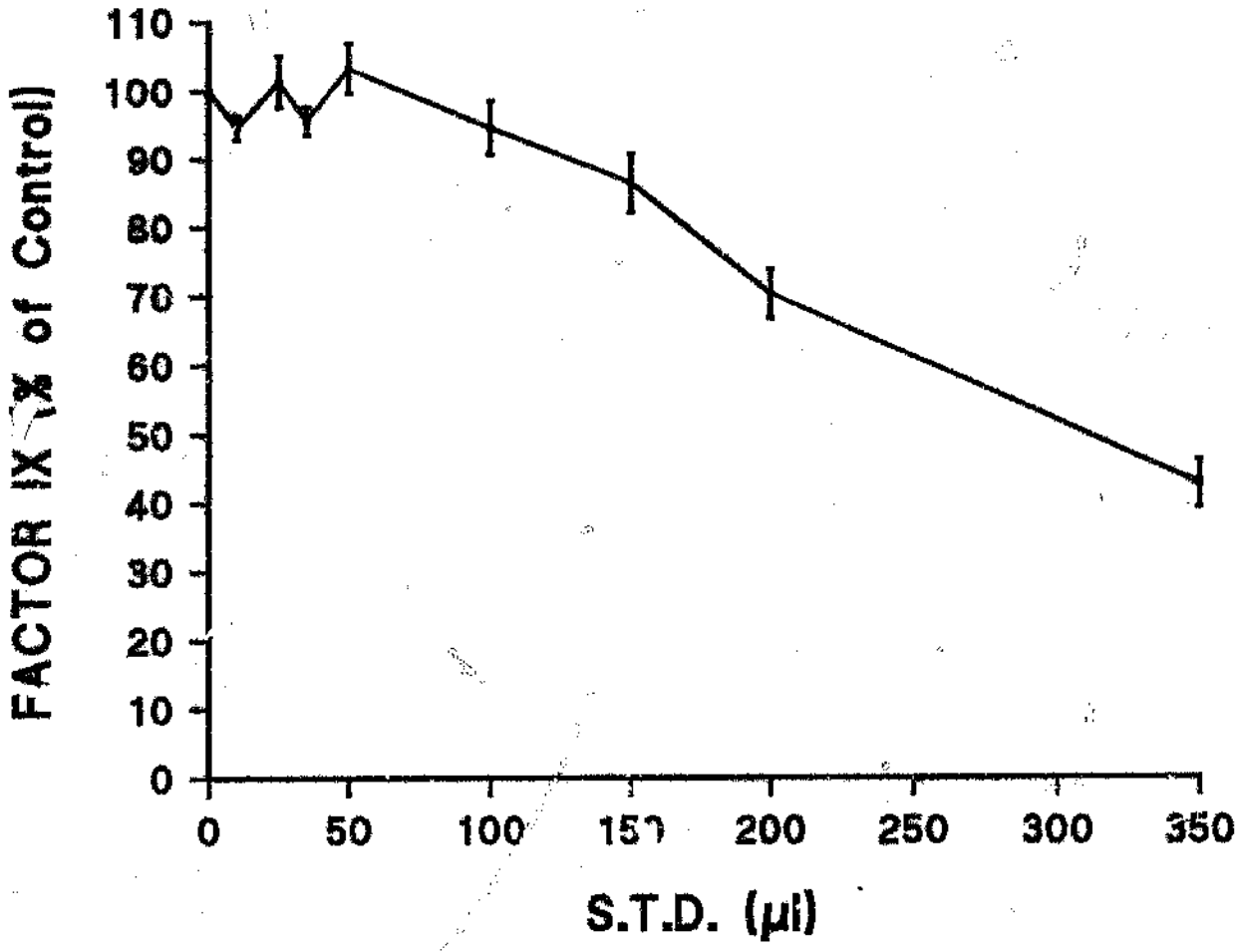


FIGURE 4.7

The in vitro effect of S.T.D. on Factor IX (Mean ± SEM)

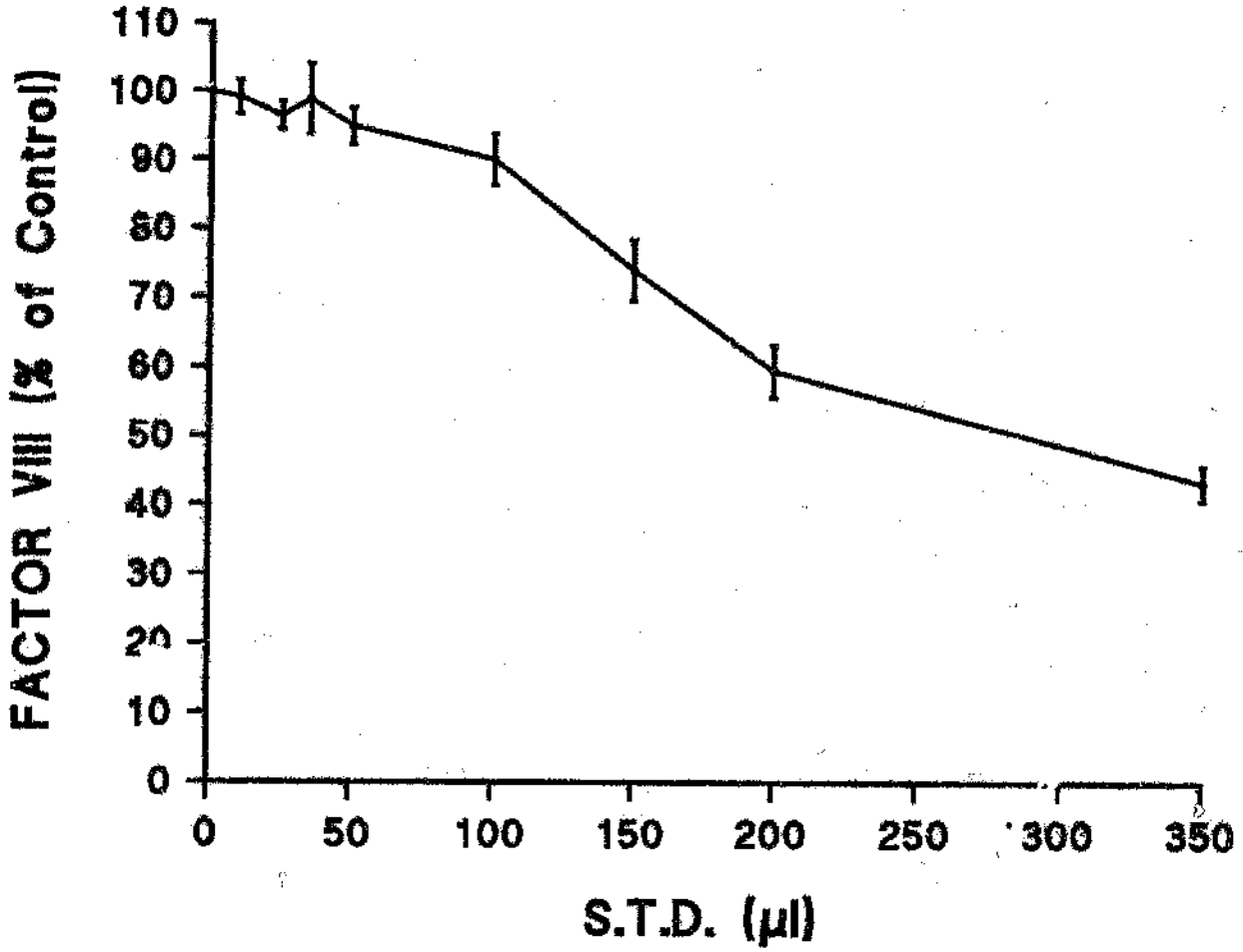


FIGURE 4.8

The in vitro effect of S.T.D. on Factor VIII (Mean ± SEM)

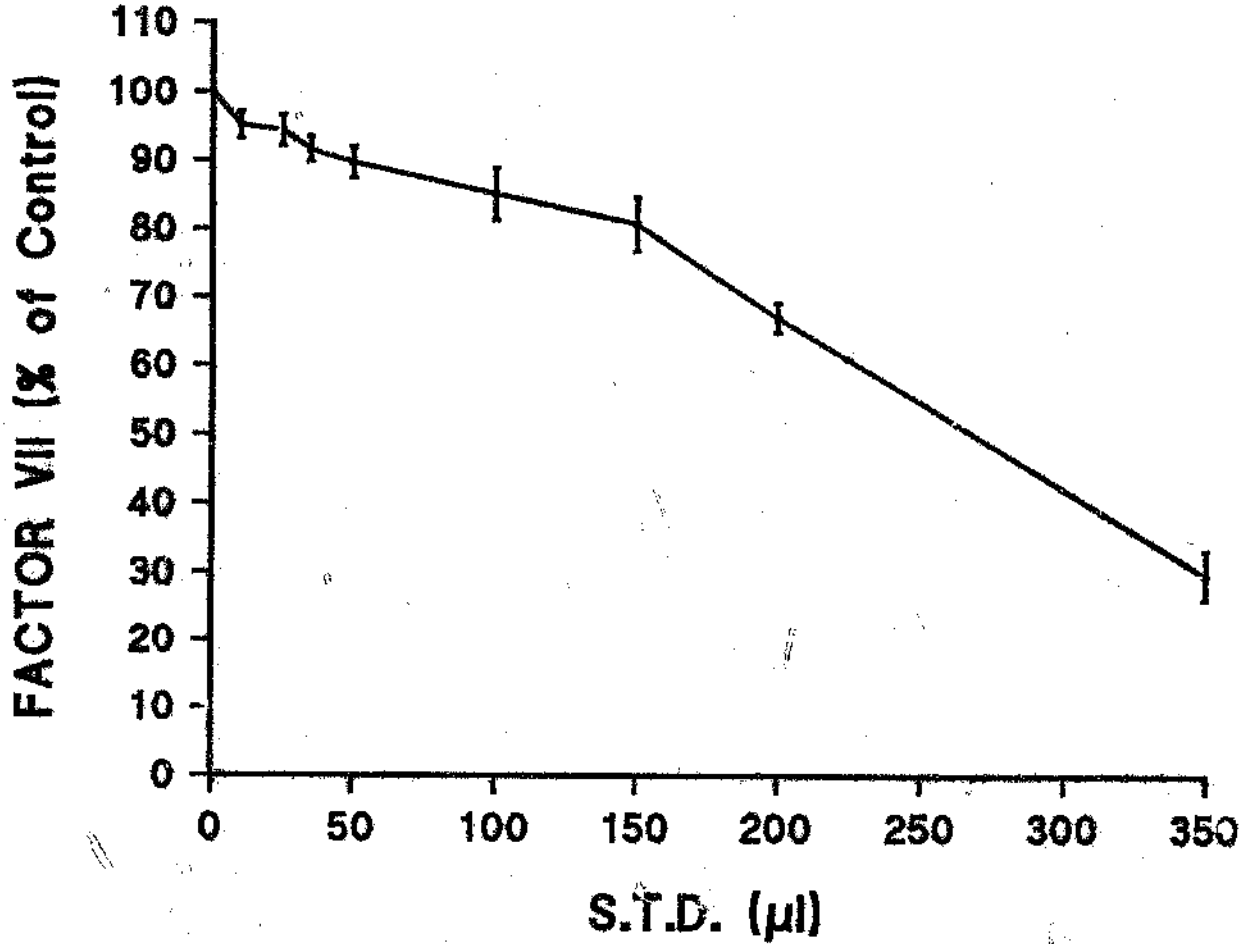


FIGURE 4.9

The in vitro effect of S.T.D. on Factor VII

(Mean ± SEM)

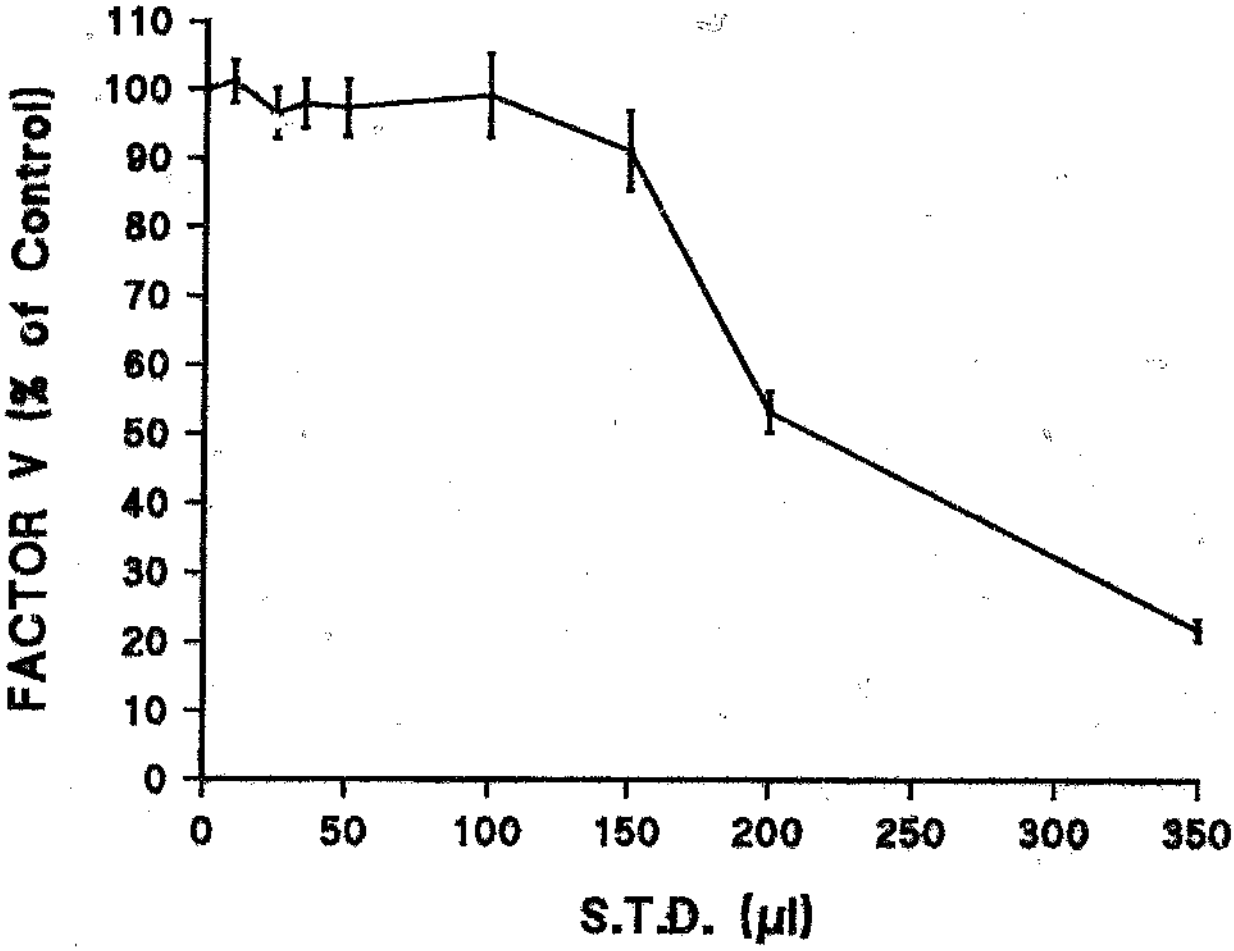


FIGURE 4.10

The in vitro effect of S.T.D. on Factor V

(Mean ± SEM)

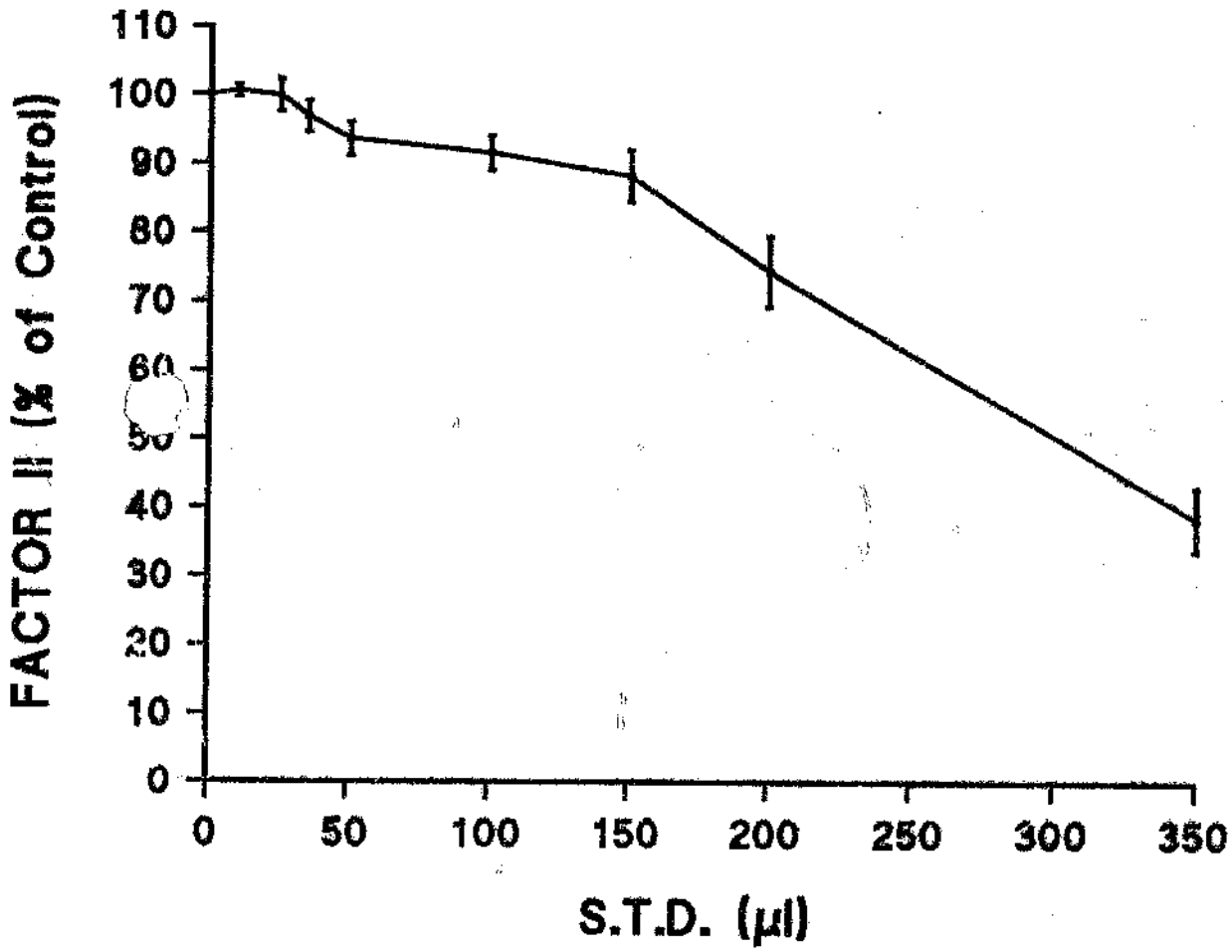


FIGURE 4.11
The in vitro effect of S.T.D. on Factor II
(Mean ± SEM)

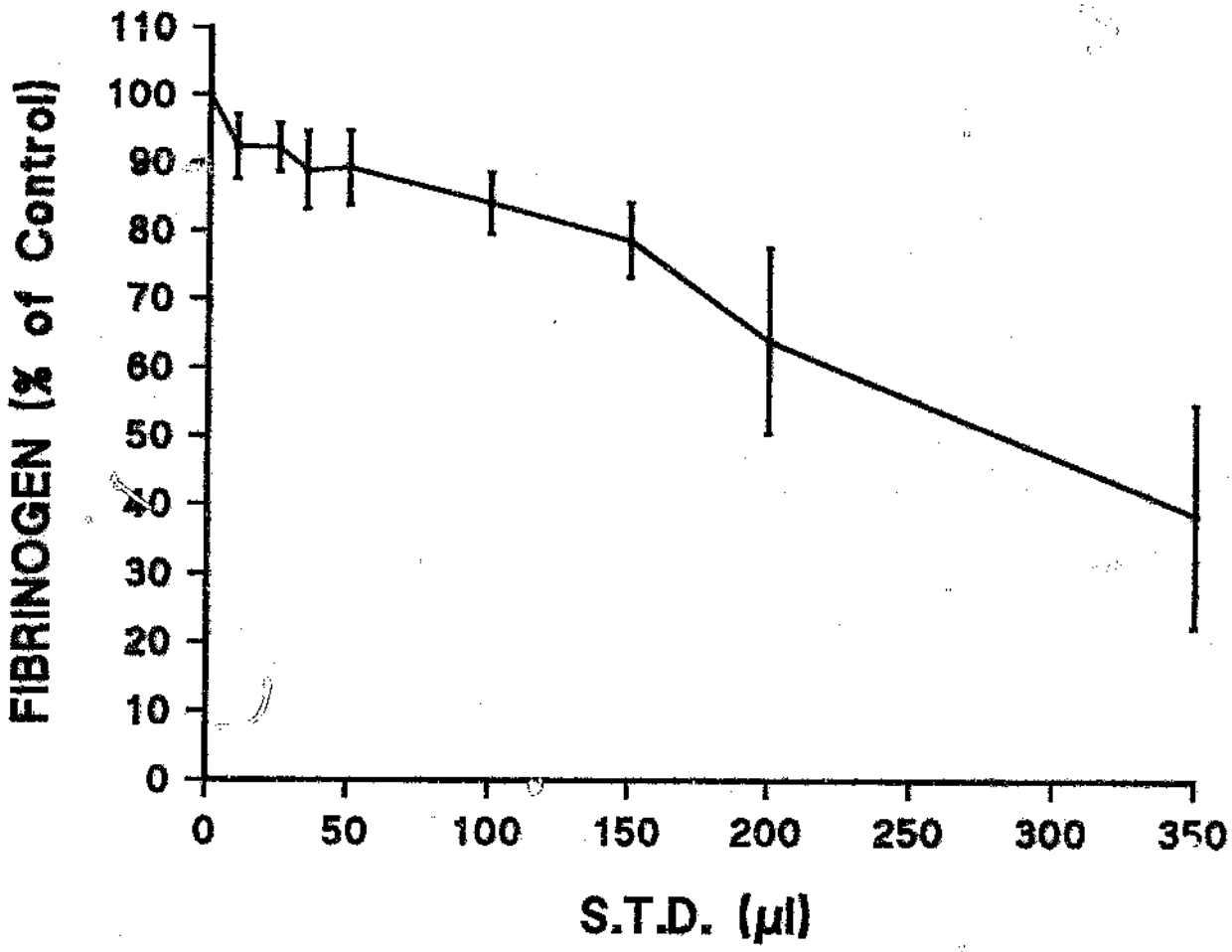


FIGURE 4.12

The in vitro effect of S.T.D. on Fibrinogen (Mean ± SEM)

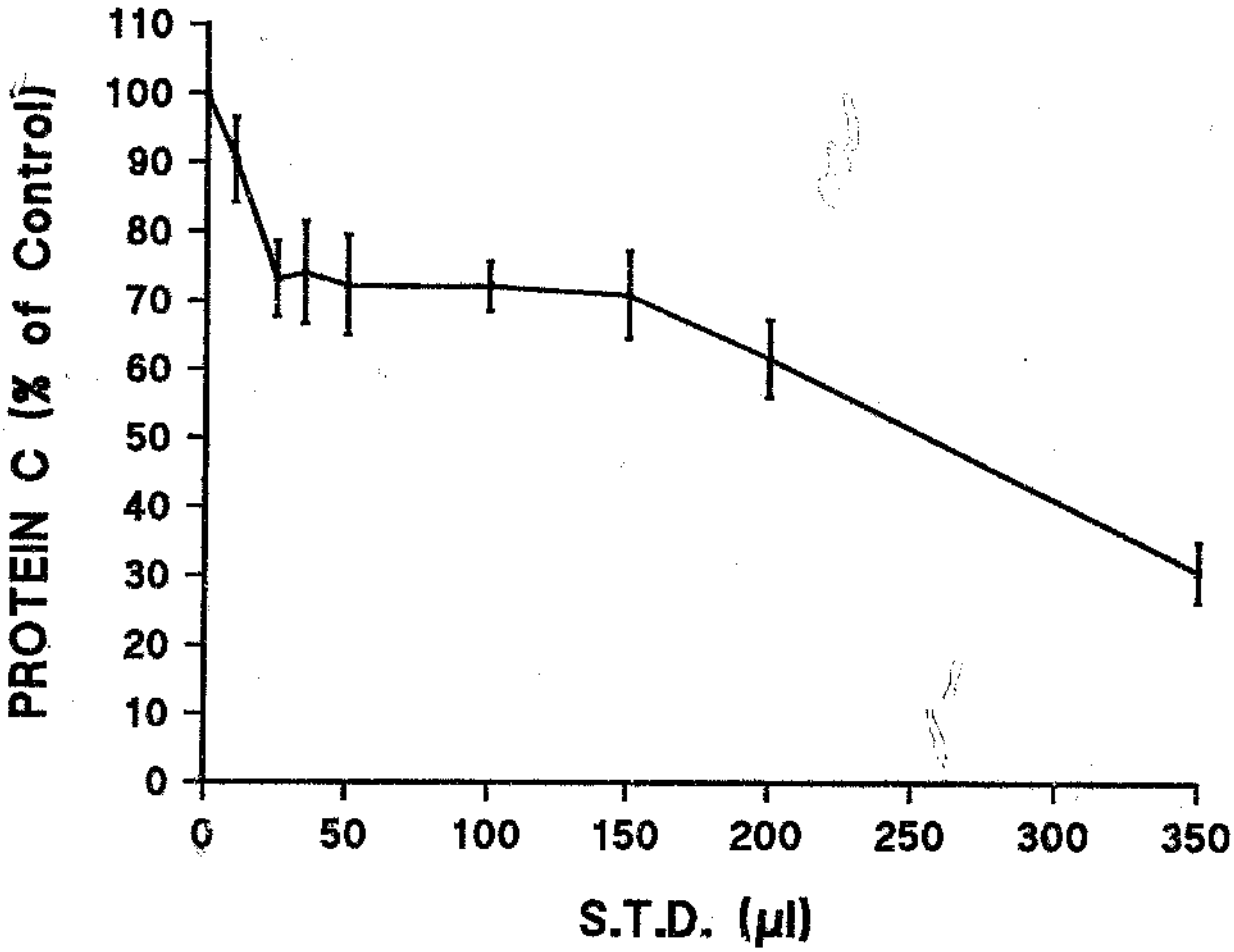


FIGURE 4.13 (a)

The in vitro effect of S.T.D. on Protein C
Note the reduction in Protein C levels
at low dosages of S.T.D.
(Mean ± SEM)

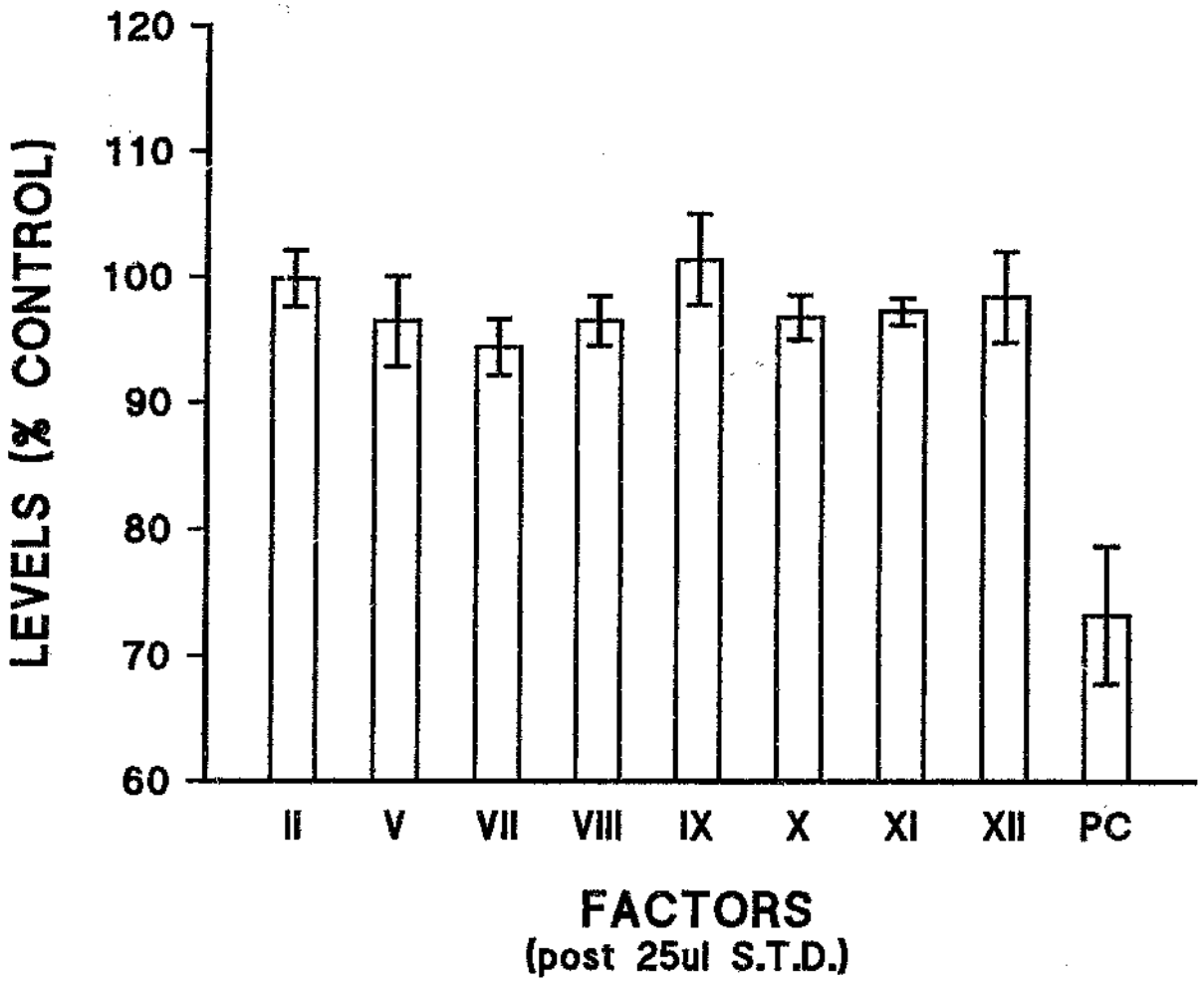


FIGURE 4.13 (b)

The level of Protein C at 25µl was signicantly less ($p < 0.001$) than the normalised levels of each of the other factors which were also not significantly different from each other

(Mean ± SEM)

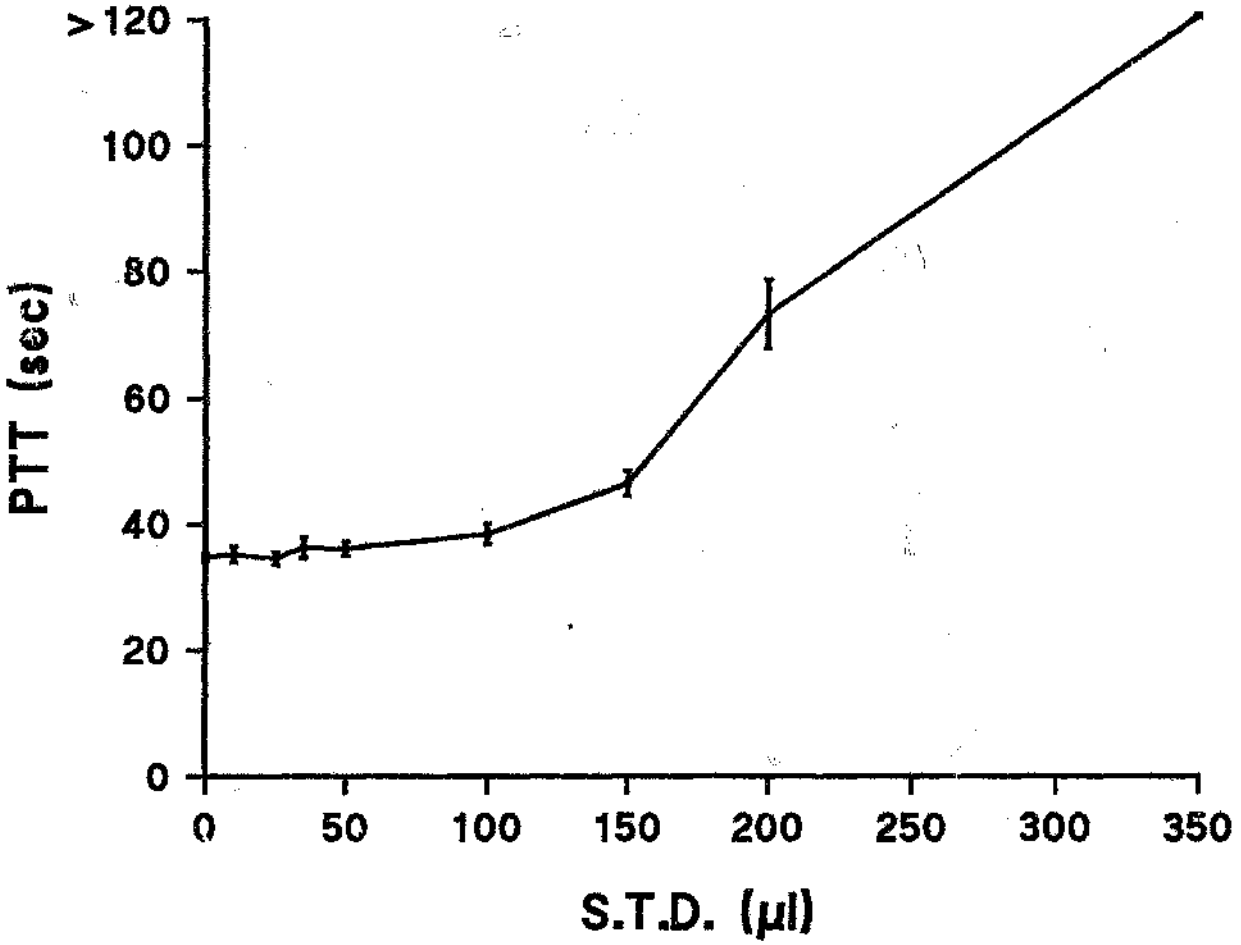


FIGURE 4.14

The in vitro effect of S.T.D. on the Partial Thromboplastin Time. Note the hypocoagulable state which occurs after addition of high dosages of S.T.D.

(Mean ± SEM)

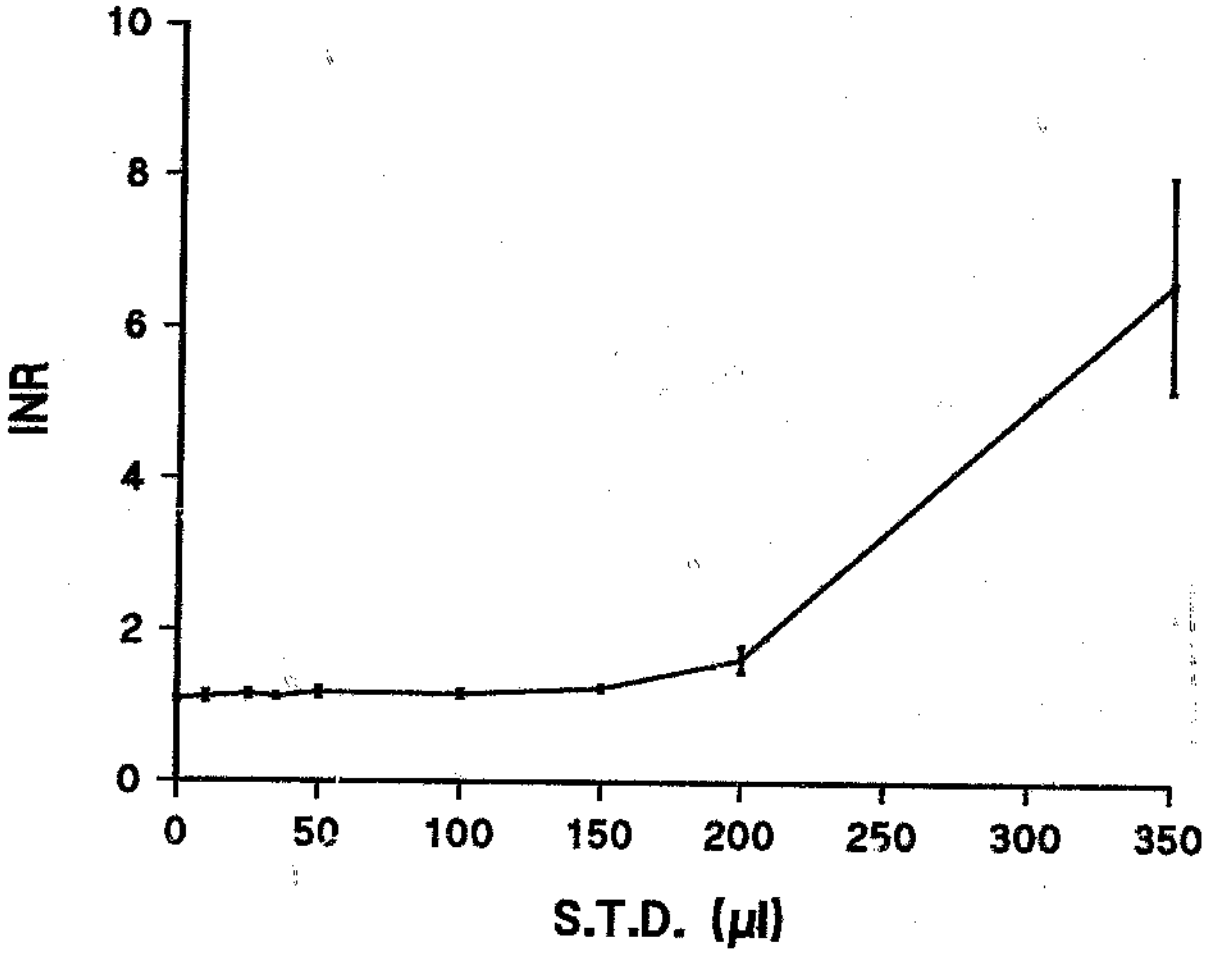


FIGURE 4.15

The in vitro effect of S.T.D. on the International Normalized Ratio (Mean ± SEM)

4.5 DISCUSSION

The finding of a hypocoagulable effect of sclerosants was originally surprising (Fegan, 1967). This finding has now been shown to be due to a decrease in activity in all of the factors of the pathway which were tested. The finding of haemolysis, which most sclerosants cause (Stroncek, 1985), was confirmed in this study. The clinical situation which is currently practised involves injection of approximately 1 ml of 3% S.T.D. into an oesophageal varix. Addition of 350 ul to 5 ml of blood results in a total incoagulable state. Therefore it is safe to assume that a similar state would occur in any varix containing less than 14.29 ml of blood.

As S.T.D. dilutes either by diffusion, or by a flow effect, a lower concentration will occur both at the site of injection and, depending on the direction and speed of the flow, this lower concentration will also occur at a site distant from the original injection site.

This low concentration would not affect any of the other factors tested other than Protein C. As previously discussed in the introduction, a depletion in Protein C is associated with a hypercoagulable state. This selective Protein C inhibition is a possible mechanism by which thrombosis occurs both at the site of injection as well as at distant sites.

The reason that the effects were not compared with blood obtained from cirrhotic patients is that while accepting that patients with oesophageal varices due to alcoholic liver cirrhosis might have coagulation abnormalities, those who bleed from their varices have these coagulation abnormalities corrected by the administration of fresh frozen plasma which contain all the relevant factors. Coagulation factors are thus normalised.

4.6 CONCLUSION

Addition of more than 200 ul to 5 ml of whole citrated blood of S.T.D. results in a hypocoagulable state. This volume is less than that used clinically, suggesting that during injection of varices a local hypocoagulable state occurs. However, at lower volumes, i.e. 25 ul, the result is selective depletion of Protein C with a probable hypercoagulable state. These findings suggested that there was scope for considerable improvement in the dosage of S.T.D. injected into oesophageal varices.

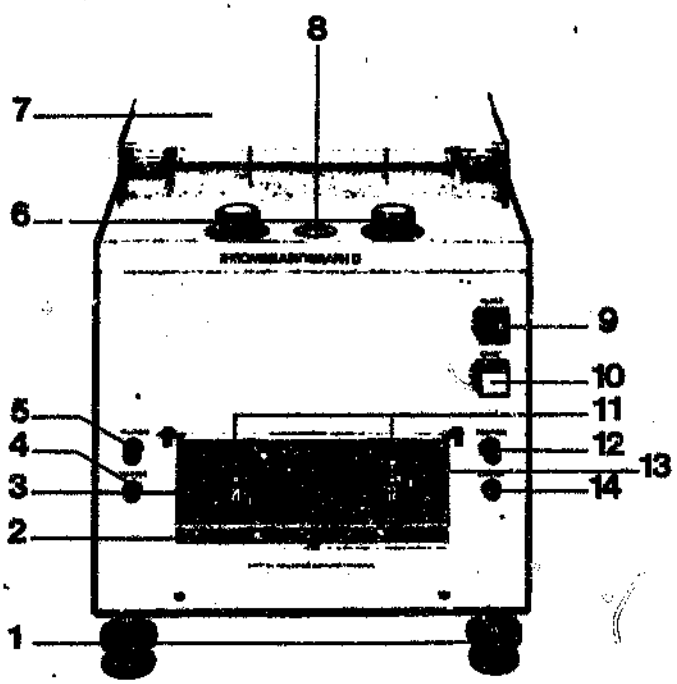
4.7 THROMBOELASTOGRAPHY

The thromboelastogram was originally developed by Hartert in Heidelberg, who first published his preliminary results in 1948. Subsequently a large amount of literature has appeared expounding the use and benefits of the thromboelastogram as a global test of the coagulum. The predominant portion of the literature has been of European nature and at present there is a relative paucity of literature from the English-speaking haematology communities. Despite this lack of enthusiasm, thromboelastography would appear to be one of the very few methods of detecting a functional hypercoagulable state (Franz et al, 1981) and the effect of Sodium Tetradecyl Sulphate was evaluated using this technique.

4.7.1 Mechanism of Action

The thromboelastogram is an optical system designed to give a continuous recording of coagulation of blood or plasma . The printout is termed the thromboelastogram. The thromboelastograph is designed with three cylindrical cuvettes of 18/8 chromium nickel steel are maintained at 37°C. An example of the instrument is shown in Figure 4.16.

Each cuvette containing the blood or plasma sample is connected to a moving device by which it is rotated back and forth over an angle of 4 degrees 45 minutes around a vertical axis. A piston composed of the same material is lowered by means of a torsion wire into the cuvette, leaving a space of 1 mm for the sample between the piston



1. Adjustable feet to level the instrument
2. Plexiglass cover to protect the measuring units
3. Two cuvettes to hold the blood samples
4. MARKER button to apply time-marking pulses for the left channel
5. Zero POSITION control for the left stylus
6. Knobs to lower and lock the pistons
7. Plexiglass cover to protect the recorder unit
8. Circular level to show when the instrument is level
9. Illuminated POWER switch (green) to turn the instrument on
10. Illuminated CHART switch (yellow) to turn the chart drive on
11. Oil pans for dampening the measuring units
12. Zero POSITION control for the right stylus
13. Cylindrical pistons
14. MARKER button to apply time-marking pulses for the right channel

FIGURE 4.16 The Thromboelastograph

and the cuvette. A mirror attached to the torsion wire reflects the light from a slit lamp as a direct visual read out.

While the sample is fluid, the pisto. is motionless. As soon as fibrin strands form between the cuvette and the piston, oscillations occur which are proportionate to the rate of fibrin formation as well as to the elasticity of the coagulum.

One oscillation requires 9 seconds for a full cycle (i.e. 3.5 seconds for each 4 degree 45 minute turn in either direction and 1 second delay at end position). The light source will reflect the oscillation so that it can be graphically recorded (Franz et al 1981).

4.7.2 Interpretation of the Thromboelastogram

(Base line = Figure 4.17)

The reaction time (r time) is a measurement from the starting point on the chart to that point where the amplitude of the oscillation has reached 1 mm. This recording is equivalent to the coagulation time or rather the recalcification time.

4.7.2.1 The r-time

The r-time has been considered by Marchal et al,(1961) to be the rate of thromboplastin generation. It will, therefore, be influenced by

changes in either generation of plasma thromboplastin or by factors of the first stage of the intrinsic pathway, i.e. factor XII, XI, IX and VIII. A lengthening of the r time will represent an inherent defect in one of the above, e.g. haemophilia A or heparin therapy. The equivalent in the test tube is a recalcification time.

4.7.2.2 The k-time

The k-time is measured from the end of the r-time to a point where the amplitude measures 20 mm. The k-time is indicative of the rapidity of the fibrin build-up or the speed with which a clot of a certain solidity is developed (Franz et al, 1981). The k-time is referred to by Marchal as the thrombin constant which is unaffected by the factors of the prothrombin complex but responsive to intrinsic plasma and platelet functions. Prothrombin depressing agents in therapeutic doses have little effect on the k-time. Prolongation of the k-time is found in deficiencies of the intrinsic factors, circulating anticoagulants and platelet disorders either quantitatively or qualitatively.

Reduction in the k-time is associated with a hypercoagulable state (Franz et al, 1981).

4.7.2.3 The ma

The ma measures the maximal elasticity of the clot. The elasticity of the clot is a function of platelet function, fibrin and all the factors relating to the fibrinolytic pathway.
The ma is converted to elasticity units (ma) by the following formula:

$$E = \frac{100\ ma}{100 - ma}$$

The ma reaches a peak and then recedes. This is thought to be a function of clot softening rather than fibrinolysis and is represented by a "spindle shaped" decline in ma.

One would therefore expect that any reduction in platelet function or count would result in a decrease in ma. Other well documented causes of a decrease in ma are circulating anticoagulants, dextran therapy and decreased fibrinogen levels. In hyperfibrinolytic states one finds a normal ma which is rapidly degraded.

4.8 METHODOLOGY

The methodology used was that described by Franz et al (1981). Four and a half millilitres of venous blood was withdrawn into a siliconised vacutainer tube continuing 0.5 ul of 3.8% sodium citrate. The various volumes of STD were now added. To recalcify the blood, a 1.29% isotonic $CaCl_2$ solution was used. A disposable tip pipette was used to pipette 0.3 ul of citrated blood into the preheated cuvette and 0.06 ul of 1.29% $CaCl_2$ was added. Special care was taken to avoid foaming. The piston was lowered into the cuvette, the specimen covered with liquid paraffin and the recording started. The tests were performed in duplicate from blood obtained from 3 healthy volunteers.

4.9 RESULTS

Examples of the actual thromboelastograms are shown in Figures 4.17 - 4.23. Of note is the shortening of the r-time and k-time with addition of between 25 ul of S.T.D. and 30 ul. Addition of larger amounts caused both the r- and k-time to prolong. Fifty microlitres of S.T.D. resulted in failure to clot. These results were reproducible.

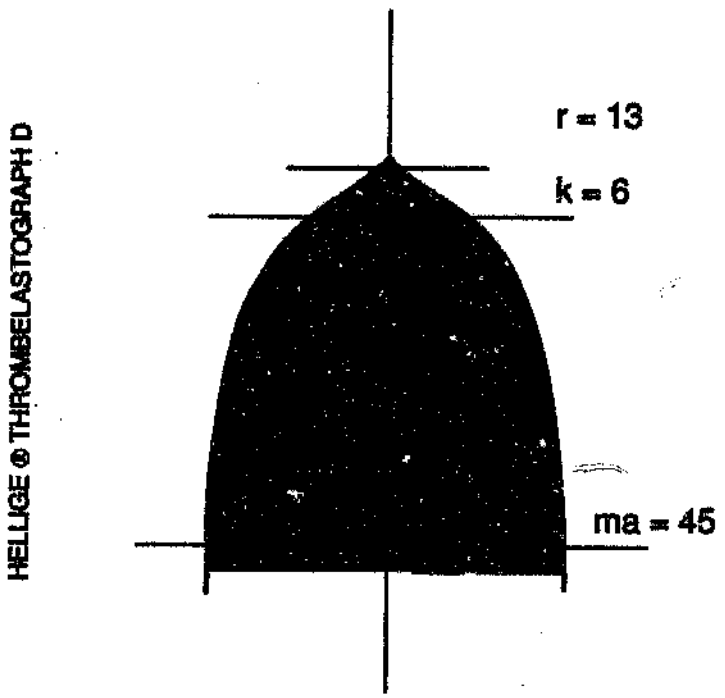


FIGURE 4.17 : A tracing of a normal T.E.G.

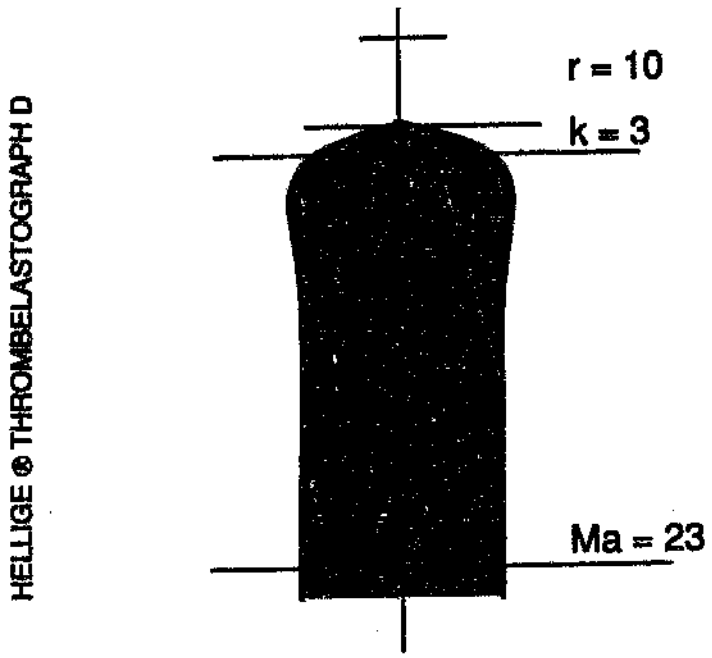


FIGURE 4.18 : A tracing of a T.E.G. after addition of 25 ul of S.T.D. Note the shortening of the r time and k time. The ma is decreased.

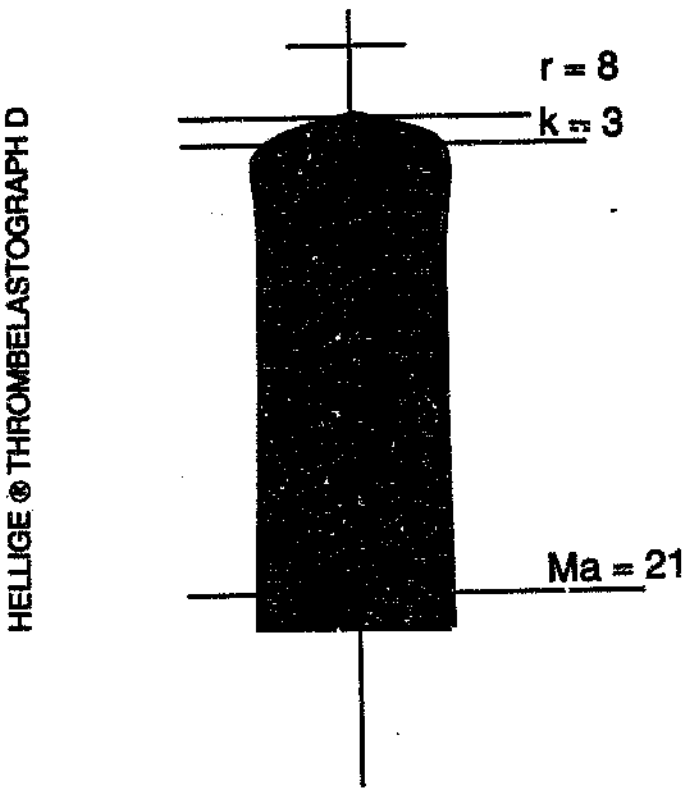


FIGURE 4.19 : A tracing of a T.E.G. after addition of 30 ul of S.T.D. The r time is further shortened.

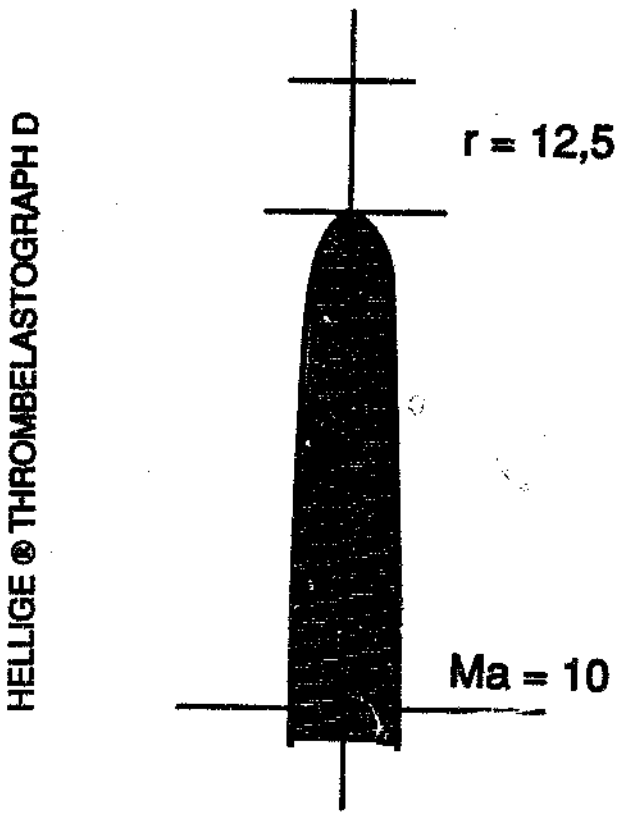


FIGURE 4.20 : A tracing of a T.E.G. after addition of 35 ul of S.T.D. The r time is now increased and the ma is further decreased.

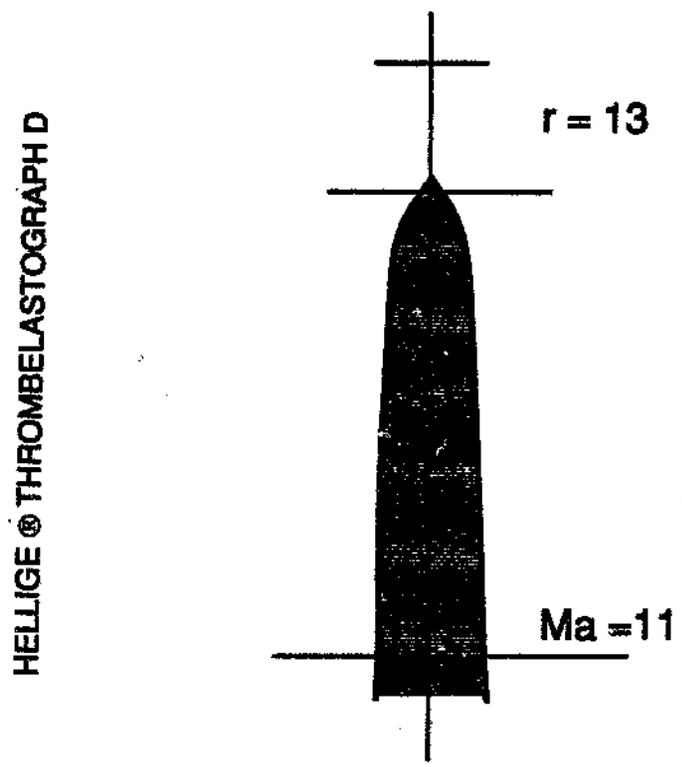


FIGURE 4.21 : A tracing of a T.E.G. after addition of 40 ul of S.T.D. The findings are relatively unchanged.

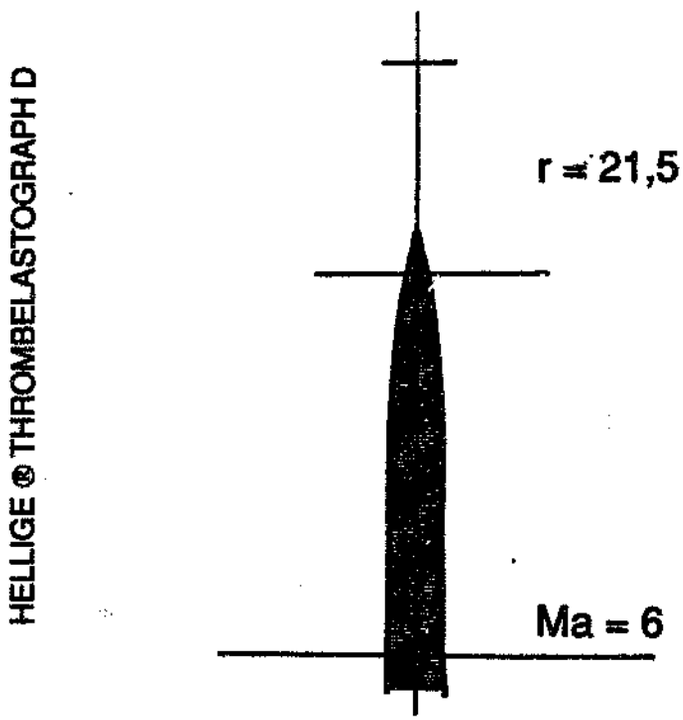


FIGURE 4.22 : A tracing of a T.E.G. after addition of 45 ul of S.T.D. The r time is now markedly increased and the ma is further decreased.

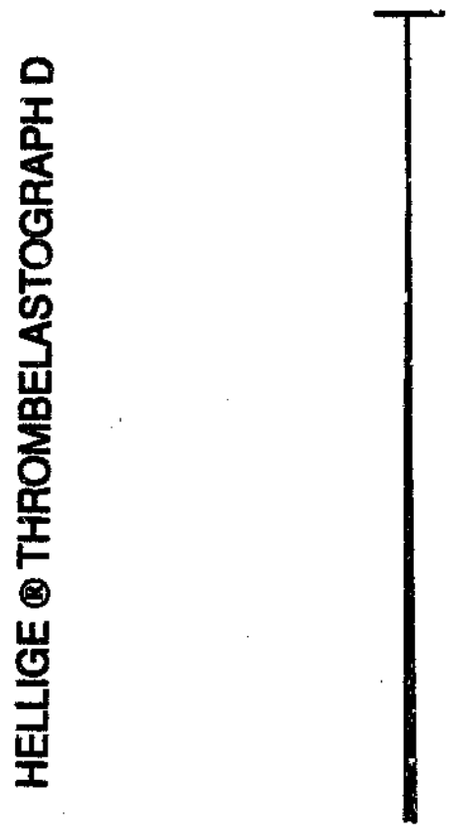


FIGURE 4.23 : A tracing of a T.E.G. after addition of 50 ul of S.T.D. The blood is incoagulable.

4.10 DISCUSSION

A hypercoagulable state is difficult to define in terms of laboratory parameters. Thromboelastograms may be one of the few methods to demonstrate the presence of a hypercoagulable state (Caprini et al, 1976). The findings of the thromboelastogram study are in keeping with the previous in vitro work, although it has not been proven that the TEG-defined hypercoagulability was the result of selective Protein C inhibition specifically.

Addition of small amounts of S.T.D. resulted in a hypercoagulable state, as evidenced by the reduction in both the r- and the k-time. The strange appearance of the slight spindle shape of the maximal amplitude might be attributed to softening of the coagulum (Franz et al, 1981). On addition of larger volumes of S.T.D., a hypocoagulable state occurred, which was in keeping with decreased levels of clotting factors.

In conclusion, the thromboelastogram provides additional evidence that a hypercoagulable state occurs on addition of S.T.D. in small volumes. Larger volumes cause a severe hypocoagulable state.

CHAPTER 5

5. ENDOTHELIUM

5.1 INTRODUCTION

The previous two chapters have evaluated the effect of S.T.D. on platelet function and coagulation factors. The cell that orchestrates thrombosis is the endothelial cell and the effect of S.T.D. on endothelium was studied. The following review is intended to highlight some new discoveries of endothelial function so that the importance of not only destruction but also injury to the endothelium is appreciated.

5.1.1 Overview

The endothelial cell plays a crucial role in determining coagulation status. Its importance has only recently been highlighted by the discovery of the numerous substances which it produces, some of which are procoagulant, while others are anticoagulant. These substances are also subject to vectorial secretion, i.e. lumenally or ablumenally. The surface area which they occupy in an average adult male is greater than 1,000 m^2 (Jaffe, 1987). The control of endothelial proliferation is very tight and while haemopoietic cell turnover for example is 5 days, endothelial turnover is measured in years (Folkman, 1987). Physiological angiogenesis occurs mainly in females during menstruation and placental development.

Angiogenesis at other times, and particularly in adult males is however generally pathological.

5.1.2 Anatomy

The endothelial cell is unique in that it lines a conduit for the blood and is the first line of cells which separate the rest of the body from the blood. The cell has a lumenal surface which under normal conditions is antithrombogenic, an ablumenal surface where it strongly adheres to the subendothelial tissue and a third cohesive surface where it binds to adjoining endothelial cells by means of cell junctions.

5.1.3 Physiology

The endothelium plays an important role in transport from the intravascular to the extravascular compartment. It is more permeable to macromolecules, water and water soluble molecules than other tissue. The mechanism of the above is thought to be due to "pores" in the cell as well as vesicles. Another mechanism is postulated to be due to the cohesive cell junctions between adjoining endothelial cells opening up.

5.1.4 Metabolic Functions

The endothelial cell has numerous functions, one of which is secretion of connective tissue components on its ablumenal surface.

This enables it to adhere to this surface. Endothelial cells have receptors on their surface for the proteins and it is thought that these interactions between receptors and these self-made proteins is vital in maintaining the cell's adhesion to the underlying subcellular matrix.

The endothelial cells respond to damage by producing vasoconstriction which aids in the process of haemostasis. This is accomplished by thromboxane A2, a prostaglandin which although found mainly in platelets, is also produced by the the endothelium. Another mechanism of vasoconstriction is as a result of adrenaline which is released in response to stress. Recently a peptide was described by Yanagisawa et al (1988) which has the ability to produce prolonged vasoconstriction. This protein, which is a short peptide, is released by the endothelial cell after exposure to thrombin and adrenaline and has been named endothelin.

The cells secrete basement membrane which differs from other collagens in that it has a high 3- and 4-hydroxyproline content. The basement membrane is a thrombogenic surface which is exposed by removal of the overlying endothelial cell. Endothelial cells also possess the ability to remodel the basement membrane via collagenases (Kalebic, 1983).

Endothelial cells which have been cultured from human umbilical veins secrete type IV procollagen, as well as laminin, fibronectin and thrombospondin. Endothelial cells in culture have been shown to secrete type V collagen into their underlying extracellular matrix (Sage, 1982). Type V collagen has been shown to inhibit endothelial cell growth.

The endothelial cells produce von Willebrand Factor (vWF) which is stored in the endothelial cells as Weibel Palade bodies. The vWF is secreted lumenally and ablumenally where it is stored in the subendothelial tissue. This enhances the thrombogenicity of this area should the endothelial cell be removed (Bloom, 1973). Prepro von Willebrand factor, which is the translational product, undergoes dimerisation and further modification before it undergoes final assembly into multimers (Ruggeri, 1987). The vWF multimers are huge and supply multiple binding sites for platelets.

The vWF factor in circulation can bind to the endothelial cells via two receptors, viz. glycoprotein Ib and the vitronectin receptor. This was shown by Asch et al (1988) in endothelial cells which had been cultured from human umbilical veins.

The platelets contain a receptor for vWF and this is thought to be the mechanism by which platelets adhere to the subendothelium. Patients with vWF disease have decreased platelet binding to the subendothelium which is corrected by the exogenous addition of vWF.

The endothelial cell, when stimulated or damaged, will act as a procoagulant. Tissue factor activity, which is normally not found on endothelial cells, is markedly increased after exposure of the endothelial cells to thrombin and especially to platelets (Johnsen et al, 1983). Factor V has been shown to be produced in endothelial cells as well as to bind to endothelial cells (Maruyama, 1984). Another mechanism by which endothelial cells localize the coagulation cascade is to bind Factors IX and X and they also activate Factor XII. Endothelial cells appear to bind stimulated platelets only. Unstimulated platelets do not adhere to endothelial cells even when the endothelial cell prostacyclin production is completely halted (Booyse, 1975). This would appear to be therefore an inherent property of the endothelial cell membrane. Stimulated platelets do bind to the endothelial cell when prostacyclin production is inhibited.

Unstimulated endothelial cells are inherently nonthrombogenic and secrete fibrinolytic agents. Prostacyclin is a non thrombogenic eicosanoid which the endothelial cell produces.

It is a prostaglandin synthesised from arachidonic acid and is secreted by the endothelial cell after the cell has been stimulated. Stimulation is effected by numerous agonists which include thrombin, high density lipoprotein, histamine, changes in shear stress (Grabowski, 1985), activated neutrophils and bradykinin (Bennett et al, 1990). It would appear that endothelial cells are not homogeneous in their ability to synthesize prostacyclin, for example endothelial cells from human foreskin capillaries synthesize other prostaglandins (Charo, 1984). Prostacyclin is a potent vasodilator as well as an inhibitor of platelet aggregation (Bennett et al, 1990). It is clinically unstable with a T ½ of 2-3 minutes and is degraded to 6-keto-PG F1 alpha. The mechanism of activation which is induced is related to a rise in c-AMP. Prostacyclin also plays a role in cytoprotection, enhanced fibrinolysis and cholesterol metabolism (Bennett et al, 1990). Removal of endothelium stops the production of all prostacyclin from exogenous arachidonic acid. Bovine aortic endothelium cells which are bovine have been shown to produce bursts of prostacyclin in response to shear stress levels (Moncada, 1987). A diet rich in fish oils of the N-3 class results in prostacyclin I_3 being formed instead of the normal prostacyclin I_2. The former is more potent in terms of its vasodilator and anti- platelet activity.

Endothelial cells also possess the ability to modify platelet function by their capacity to metabolize ADP and ATP to adenosine and adenosine monophosphate which inhibit platelet aggregation. In contrast when damaged they release ATP which results in platelet aggregation (Pearson, 1979). Antithrombin III is a plasma protein whose action is to inactivate thrombin. This action is greatly accelerated by endothelial cells which have the ability to bind heparin to their surface (Jaffe, 1987).

Protein C is activated by thrombin which is bound to thrombomodulin. Thrombomodulin is an unusually acidic protein with a pH of 2.5 and is found on endothelial cell surfaces. The mechanism of action would appear to be a complicated relationship between Factor Va and thrombomodulin. Depending on absolute relative concentrations of these two cofactors, protein C activation can be either enhanced or inhibited. In the presence of a low concentration of Factor Va, protein C activation is accelerated and recirculated back to the source of thrombin thereby dampening the signal. However in the presence of Factor Va in high concentrations, thrombomodulin will be depressed and coagulation will be accelerated. (See Fig 5.1)

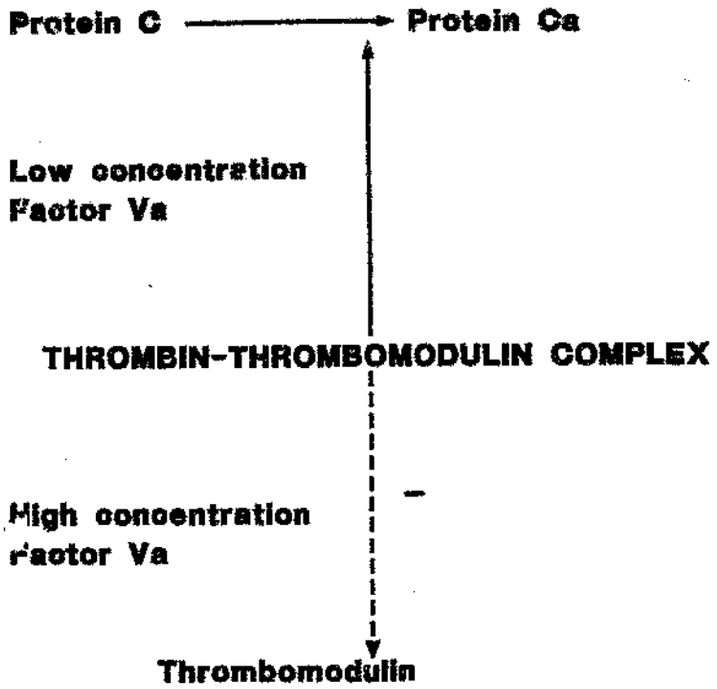


Figure 5.1

A simplified diagram showing that thrombin is inactivated by complexing with thrombomodulin where there is a low concentration of Factor Va. This complex results in Protein C activation. However, in the presence of a high concentration of Factor Va, thrombomodulin is depressed allowing thrombin to remain activated and accelerate thrombosis.

Endothelial cells have been shown to secrete plasminogen activator in response to thrombin (van Hinsburgh, 1985) and therefore are also intimately involved in the fibrinolytic pathway. Endothelial cells secrete a growth factor , endothelial cell derived growth factor (ECDGF), which is very similar to platelet derived growth (PDGF).

The endothelial cell produces endothelial derived relaxing factor. This substance has been recently identified as nitric oxide (NO) (Palmer, 1987). Endothelial derived relaxing factor has been shown to produce vasodilatation and inhibits platelet aggregation as well as platelet adhesion to endothelial cells (MacDonald, 1988).

Endothelial cells produce platelet activating factor (PAF) in response to numerous agonists which include thrombin and vasopressin (McIntyre, 1985), Endothelial cells secrete platelet derived growth factor (PDGF) ablumenally. PDGF binds to receptors on fibroblasts and smooth muscle cells and is mitogenic for these cells (Ross, 1986).

5.2 MATERIALS AND METHODS

5.2.1 Endothelial Vein Modal

Umbilical cords were obtained immediately after the second stage of delivery. All cords were from patients who did not have foetal distress, and the cords were trimmed to remove damage caused by clamps. The umbilical vein was cannulated with a No 23 Jelco catheter and the vein was then perfused for 2 minutes with normal saline at a constant pressure (20 mm Hg) to emulate variceal pressure. The experimentation on the umbilical cords was performed at 25 C. The flow rate was standardised at 100ml/min. The sclerosant was injected freehand using a 2ml syringe in the same manner applied in the clinical situation.

Cord lengths varied from 12cm to 20cm. Before the experiments using the sclerosant were performed, saline was used as a control in seven cords to document that no endothelial damage occurred. One millilitre of the sclerosant (S.T.D.) was injected. The concentrations injected were 3% m/v (undiluted), 1/10 dilution and 1/100 dilution. The veins were then reperfused with normal saline for 5 minutes. The S.T.D. perfusions were performed on three separate cords. This was repeated for each dilution used.

To determine whether binding of the sclerosant to blood would alter the effect on the 1:10 dilution of STD, the experiment was repeated using whole blood as the perfusant. The cords were then sectioned at 1 cm intervals and then fixed in glutaraldehyde and submitted for light as both scanning and transmission electron microscopy. The light microscopy specimens were also subjected to Factor VIII staining.

5.2.1.1 Transmission Electron Microscopy

One millimetre blocks of tissue were placed in glutaraldehyde fixative. The tissue was fixed overnight (24 hours), and rinsed for 10 minutes in Millonigs phosphate buffer. The tissue was postfixed in 1% osmium tetroxide for 1 hour and then rinsed for 10 minutes.

The tissue was rinsed with 50% alcohol and then left for 10 minutes in 50% alcohol. This process was repeated using 75% alcohol, followed by 100% alcohol. It was then left for a further 20 minutes in 100% alcohol, following which it was rinsed with propylene oxide and then left for 20 minutes in propylene oxide. It was then placed for 10 minutes in 50% resin/propylene oxide followed by a further 10 minutes in 75% resin/ propylene oxide, followed by 100% resin for 10 minutes. It was then re-imbedded in fresh 100% resin.

The resin was then polymerized for 3 hours at 100°C in an oven. The tissue was cut in thick sections (1 micron) and stained with azure II.

Area to be examined was selected by using light microscopy. Thin sections (90 nm) were then cut and were picked up on copper grids. The grids were stained with saturated uranyl acetate and lead citrate. The specimens were viewed on the transmission electron microscope - Hitachi H-600.

i) Glutaraldehyde fixative was made up by adding 90 ml Buffer and 10 ml 40% formaldehyde. Three ml of this mixture was discarded and 3 ml of 25% EM grade glutaraldehyde was added.

ii) Millonigs Phosphate Buffer was made up by adding 1000 ml water, 18.8 g sodium dihydrogen phosphate and 4,2 g sodium hydroxide.

5.2.1.2 Scanning Electron Microscopy

One millimetre blocks of tissue were fixed in 33% glutaraldehyde fixative for 24 hours. They were then rinsed in Millonigs phosphate buffer for 10 minutes, post fixed in 1. osmium tetroxide for 1 hour and rinsed again for 10 minutes. The tissue was rinsed with 50% alcohol and then left for 10 minutes in 50% alcohol. This was repeated using 75% alcohol, and then 100% alcohol.

The specimens were then left to stand for a further 20 minutes in 100% alcohol. The tissue was then critically point dried. The tissue was attached to stubs for the scanning electron microscopy with a thin layer of colloidal graphite. The stubs were then coated in the sputter coater. The specimens were viewed on the scanning electron microscope.

5.2.1.3 Immunoperoxidase Staining of Umbilical Cords

The sections were cut transversely 3-4 um and placed in a 50°C incubator overnight. The slides were brought to water, viz. xylene for 20 minutes, followed by absolute alcohol and then rectified spirits. The slides were then placed in 0.1% trypsin in phosphate buffered saline (prewarmed) for 15 minutes at 37°C to unmask the antigenic sites. The slides were well washed in tap water to stop the reaction. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 30 minutes. The slides were then washed 3 times in phosphate buffered saline and were placed in an incubating chamber. The background stain was blocked with 10% normal horse serum for 20 minutes. The slides were drained and wiped around each section and incubated with rabbit anti-human von Willebrand Factor antibody which was diluted 1:100.

This antibody was supplied by Dakopatts (A082). The slides were incubated at room temperature for 60 minutes. The slides were then rinsed well in phosphate buffered saline, drained and wiped around the section. The slides were then incubated with secondary antibody, which was anti-rabbit immunoglobulin biotinylated species specific whole antibody from donkey, for 30 minutes. This secondary antibody was supplied by Amersham (RPN 1004) and was diluted 1:200. The slides were then again washed well with phosphate buffered saline, drained and wiped around the section. The slides were then incubated for 30 minutes in streptavidin biotinylated horseradish peroxidase complex dilution 1:300 (RPN 1501). The slides were washed well with phosphate buffered saline and placed in coplin jars. The slides were incubated in peroxidase substrate solution for 5 minutes. They were then washed with running tap water for 5 minutes. The slides were then counterstained with Mayer's haematoxylin for 3 minutes. The slides were then blued by placing them in water and dehydrated by placing them in rectified spirits, followed by absolute alcohol and then xylene. Finally, the slides were mounted.

i)Phosphate Buffered Saline

The phosphate buffered saline was made up by adding 8 grams of sodium chloride, 1.15 grams of disodium hydrogen phosphate, 0.2 grams of potassium chloride and 0.2 grams of potassium dihydrogen orthophosphate to 1 litre distilled water. The pH of the solution was then adjusted to 7.6

ii)Peroxidase Substrate Solution

The peroxidase substrate solution was made up by adding 0.1 ml of 3,4,3'4' tetra-aminobiphenyl hydrochloride, 3.3' diaminobenzidine tetrahydro- chloride (BDH), 100 ml of phosphate buffered saline, and 0.003% 3 drops hydrogen peroxidase in 100 ml distilled water. Equal quantities were added together immediately before use.

5.3 RESULTS

A 1/100 dilution of S.T.D. caused no detectable damage. Damage was assessed as absence of endothelial cells. A 1/10 dilution limited the extent of denudement to 8 cm downstream in two of the cords and 7cm in one of the cords. The undiluted S.T.D. resulted in complete destruction of the endothelial layer for the entire length of the vein.

Figure 5.2 demonstrates the effect of perfusion of normal saline for 5 minutes and is the control. The endothelium is intact. This is also shown on scanning electron microscopy (Figure 5.4.) and transmission electron microscopy (Figure 5.6). von Willebrand antibody was shown to bind both to the endothelial cells as well as the subendothelial tissue (Figure 5.8)

Figure 5.3 demonstrates the effect of 1/10 dilution S.T.D. at 6 cm. The endothelium is completely destroyed. All that remains at the site are ghosts of amorphous material. This was confirmed on transmission electron microscopy (Figure 5.7). Scanning electron microscopy (Figure 5.5) demonstrated the exposure of the underlying collagen. The remaining subendothelial tissue was still shown to bind to Factor VIII antibody (Figure 5.9). The findings of the extent of damage were unaltered when the saline perfusant was replaced with blood, viz 8 cm.

FIGURE 5.2 : Photomicrograph demonstrating normal endothelium lining a human umbilical vein.

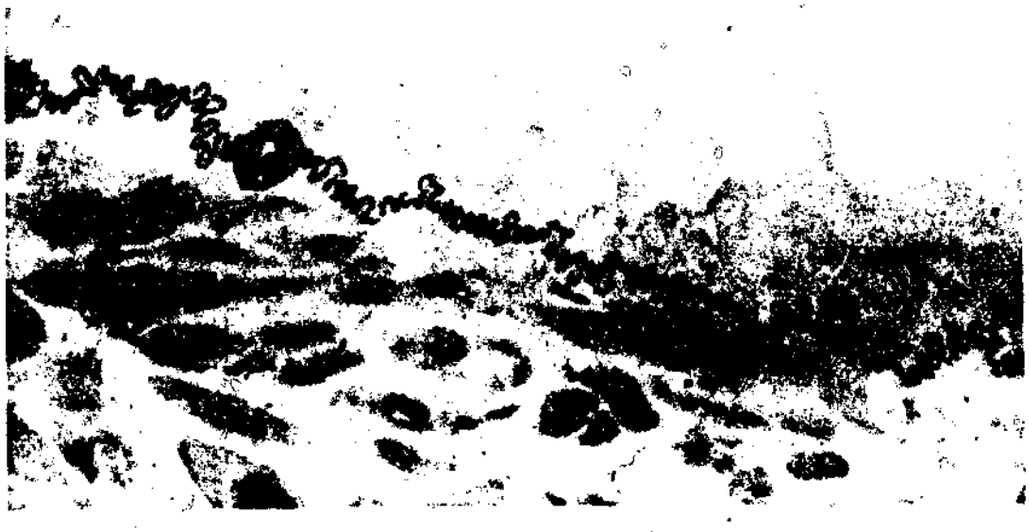


FIGURE 5.3 : Photomicrograph demonstrating destruction of the endothelial layer after perfusion with S.T.D. The ghosts of the cells are visible.

FIGURE 5.4 : Scanning electron micrograph demonstrating normal endothelial surface.

FIGURE 5.5 : Scanning electron micrograph showing denudement of the endothelial layer and exposure of collagen in the subendothelial layer.

FIGURE 5.6 : Transmission electron micrograph showing a viable endothelial cell. A normal tight junction is demonstrated.

FIGURE 5.7 : Transmission electron micrograph showing cellular debris after perfusion with S.T.D.

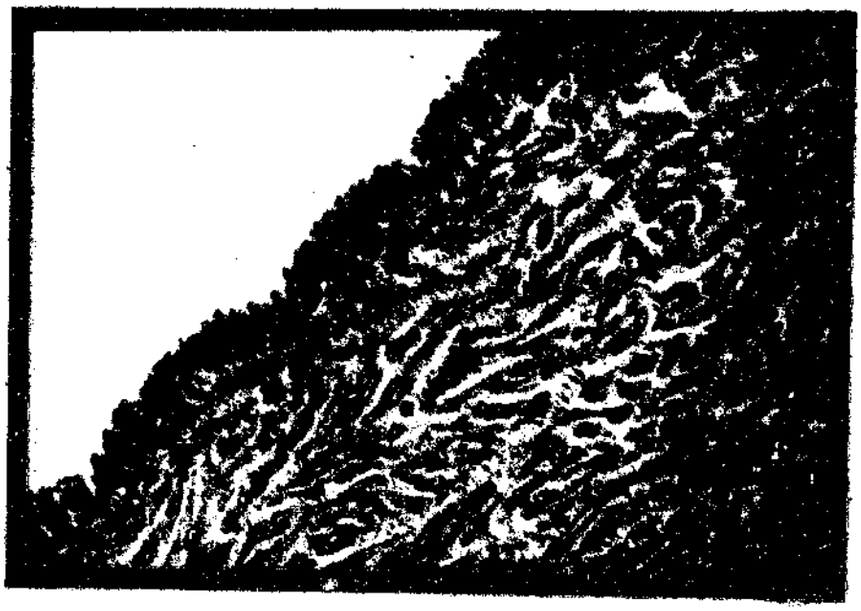


FIGURE 5.8 : A photomicrograph demonstrating von Willebrand Factor binding to endothelium and subendothelial tissue.

FIGURE 5.9 : A photomicrograph demonstrating the von Willebrand Factor still binding to the subendothelium after the endothelium had been destroyed by perfusion with S.T.D.

5.4 DISCUSSION

A human umbilical vein model was devised. The validity of the endothelial function of endothelial cells obtained from human umbilical veins has been documented by Jaffe et al, (1973). They used a culture system and subsequently most of the work on the metabolic functions of endothelial cells has been documented using this tissue culture technique. Fegan et al, in 1965, showed histologically that S.T.D. is toxic to the endothelium, while Stroncek et al (1985) confirmed their findings using cultured endothelial cells. An in vivo model, the dorsal vein of a rat's tail, was used by Blenkinsopp (1968) This model is more physiological perhaps than cultured endothelial cells, but it is difficult to quantify the extent of damage produced by different dosages of sclerosant due to the small size and length of the vein. Another criticism is that the model is not human.

In considering important factors applicable to the design of a suitable model for investigating varices, pressure, temperature and flow rate came to the fore. A pressure of 20 mm Hg was standardised to emulate intravaricel pressure. The experimentation on the umbilical cords was performed at 25 C. Apart from convenience, the reason for choosing room temperature was the fact that the varices of patients who undergo oesophageal endoscopy are subjected to flows of both air and water at room temperature. This is especially crucial when the endoscopist is evaluating bleeding varices.

Flow of blood in varices would appear to be extremely variable. McCormack et al (1983) developed an ultrasonic probe which was able to detect a minimum flow rate of 11 ml/min. They were able to show that the flow in a varix varied with the position of the patient and that it increased during inspiration and decreased with expiration. In some cases there was flow in both directions. Furthermore, general anaesthesia and positive pressure ventilation resulted in a reversal of previous flow direction. Giani et al (1990) confirmed the variability of direction of blood flow in varices.

In view of the wide variation in variceal blood flow experienced in vivo, no in vitro model can faithfully represent in vivo flow patterns. However, this problem is by no means unique to the investigation of oesophageal varices. Indeed it is common to all vitro experimentation, of whatever nature, that not all the conditions prevailing in vivo can be reproduced faithfully in vitro. Despite this limitation, there are numerous examples of in vitro technology having contributed enormous insight into the in vivo physiology which it so imperfectly represents.

For instance, the discovery, biochemical characterisation and ultimately the production for pharmaceutical purposes of the eighteen recombinant haemopoietic growth factors and interleukins currently available, was entirely dependent on in vitro bioassays, which were very poor representatives of conditions within the bone marrow (Moore, 1991). Furthermore, organ culture systems, employing pieces of tissue removed from the body and observed in isolation for biological responses to various agents, included the seminal work of Vane (1971), which culminated in a joint Nobel Prize in physiology in 1982.

For the purposes of this study, flow rate was standardised at 100 ml/min for the umbilical vein model. The majority of blood flowing through gastro-oesophageal varices terminates in the azygous vein system (Sherlock, 1990) and flow rate in the azygous system is therefore thought to represent a good index of flow through varices (Bosch et al, 1988). Azygous flow rates in patients with alcoholic liver cirrhosis who have bled have been measured at approximately 600 ml/min (Sherlock, 1990). Bearing in mind that the parallel network of varices which ultimately terminate in the azygous vein, it was decided empirically to standardise the flow at 100 ml/min. One has to acknowledge that flow rates of greater than 100 ml/min in any one varix would limit endothelial damage.

However, given the variability which occurs in variceal blood flow on inspiration and expiration, indeed even to the point of reversability in some cases, it seems unlikely that a flow rate of more than 100 ml/min (1/6 of total azygous flow would be exceeded in any one varix for any length of time. Indeed one would expect that the model would overestimate rather than underestimate the extent of damage.

The perfusant used was normal saline. This was chosen based on the work of Mazer et al (1986), who showed that normal saline was the least damaging of irrigating solutions on the endothelium of small arteries of rats. In an effort to render the experiment more physiological, the 1/10 dilution experiment was repeated using blood as the perfusant with identical results. An advantage of the model is that the umbilical cord also contains two arteries, so that the endothelium of the arteries could be used as a control to exclude an extraneous cause of endothelial damage. A final advantage is that with the current opposition to vivisection, this model bypasses the problem of animal experimentation.

The umbilical vein model confirmed that the sclerosant S.T.D. destroys endothelium. The contribution of the model has been to document the extent of damage.

A disadvantage of the model is that it only assesses the ability of the sclerosant to denude the vessel of its endothelium and despite showing that von Willebrand Factor is still present in the subendothelium, the von Willebrand Factor antibody does not assess whether this procoagulant protein is still functional. Despite this disadvantage, one would expect that if removal of the cell is the endpoint, then there must be a gradient where, although the cell is damaged, it is still present. This would occur in decreasing frequency as the concentration of the sclerosant decreased or in other words distal from the point of injection. Removal of the endothelium and the subsequent exposure of the highly thrombogenic subendothelial surface would undoubtedly lead to thrombus formation if enough procoagulant protein activity as well as functional platelets were present. What is noteworthy, is the extent of damage that the undiluted sclerosant produced. This is in keeping with the postmortem findings of Evans et al (1982) who showed damage at considerable distances from the site of injection. The 1/10 dilution of S.T.D. limited the extent of damage to 8 cm which is closer to what is desired in the clinical situation.

From the introduction, it is noted that the physically present endothelial cell acts in a highly thrombogenic manner when damaged and therefore one would expect that the thrombus formation would propagate for some distance distal to the site where absence of endothelium was noted.

The findings of previous chapters suggested platelet hyperaggregability and hypercoagulability from dilute S.T.D., as opposed to platelet lysis and incoagulability from standard dosage S.T.D. When considered in relation to the findings of this chapter, these features suggested that 1/10 diluted S.T.D. should actually be more thrombogenic locally than S.T.D. in standard dosage, and would have the added bonus of limiting extensive and possibly systemic side effects and complications. This was the impetus to test the hypothesis in the clinical situation both for acutely bleeding oesophageal varices, as well as for preventing recurrent bleeds.

CHAPTER 6

6. EFFECTS OF STANDARD DOSAGE VERSUS DILUTE SODIUM TETRADECYL SULPHATE ON OESOPHAGEAL VARICES: A PROSPECTIVE RANDOMISED PILOT STUDY

6.1 INTRODUCTION

Sclerotherapy of oesophageal varices is widely accepted as a technique of choice to thrombose the acutely bleeding varix and for long term variceal obliteration (Terblanche et al, 1990; Sivak et al, 1990). Various sclerosants are currently available and would appear to be of major importance in determining the efficacy and complication rates for the procedure. The reason for choosing S.T.D. has been previously discussed.

Blenkinsopp, in 1968, using a rat vein model demonstrated the superiority of intravenous injection of 3% Sodium Tetradecyl Sulphate (S.T.D.) as compared with other agents. Subsequent studies have shown its efficacy is comparable with ethanolamine oleate (Chung et al, 1988; Kitano et al, 1988), but concern has been expressed as to the local complications which occur subsequent to S.T.D. administration (Terblanche et al, 1990).

Substantial dilutions of S.T.D. were shown in Chapter 5 to be effective in stripping vascular endothelium yet were not associated with incoagulability of blood nor with platelet lysis (Chapters 3 and 4).

Indeed dilute S.T.D. was found to actually induce platelet aggregation and to contribute towards hypercoagulability of blood through a selective low dose inhibition of the physiological anticoagulant protein C. These findings suggested that treatment with ten fold diluted S.T.D. may be as effective as, if not more effective than, standard dosage treatment, yet probably less prone to ulcerative complications and their sequelae.

A pilot study was therefore initiated in which patients with portal hypertension and oesophageal varices were randomised prospectively into standard dosage versus dilute S.T.D. treatment arms.

6.2 METHODS AND PATIENTS

Fifteen consecutive patients receiving sclerotherapy at the Johannesburg Hospital over a 6 month period were included in this study. There were 9 patients in Group A and 6 in Group B. 4 of the patients in Group A presented with an acute index bleed while only 3 in Group B had this problem. All the remaining patients from both groups were on chronic sclerotherapy for a previously documented variceal bleed. None of the patients presented with an interval bleed.

There were 5 males in Group A vs 4 in Group B. The aetiology in both groups included Laennec's cirrhosis in 7, primary biliary cirrhosis in 1, chronic active hepatitis in 3, portal vein thrombosis in 3 and idiopathic in 1. Their details are presented in Figure 6.1. All patients signed consent and the project was approved by the Ethics Committee of the University of the Witwatersrand.

The patients were randomised prior to detailed assessment into receiving dilute sclerosant (Group A), which was a 1/10 dilution of 3% m/v S.T.D. and undiluted sclerosant, 3% m/v S.T.D. The endoscopists and patients were blinded as to the nature of Group A and Group B. The dilution was made up by the Pharmacy Department of the Johannesburg Hospital. The technique used was injection via a flexible gastroscope. The varices were injected intravariceally.

	GROUP A	GROUP B
Sex (male/female)	5/4	4/2
Age (years) range	36 - 78	18 - 69
Median	49	47.5
Child-Pugh Classification		
A	5	1
B	2	4
C	2	1
Median volume injected (ml)	8.3	7.3
Range volume injected (ml)	5 - 10	4 - 12
Mean follow up (months)	7	6
Aetiology		
Alcohol	5	2
Primary Biliary Cirrhosis	1	-
Chronic Active Hepatitis	1	2
Portal Vein Thrombosis	2	1
Idiopathic	-	1

FIGURE 6.1 : PATIENT PROFILE.

	GROUP A	GROUP B
Deaths	1/9	1/6
Rebleed rate (to date)	0/8	3/6*
Efficacy in stopping		
Acute bleed	5/6	3/3
Objective endoscopic		
improvement of varices	8/8	5/5
Ulceration rate	1/8	6/6
Stricture	0/8	4/5

* 2/6 were due to ulceration.

FIGURE 6.2 : EFFICACY AND MORTALITY

6.3 RESULTS

Results of treatment in the 2 groups are summarized in Figure 6.2. There was one death in each of the groups. The patient from Group A had an acute cardiac arrest 6 hours after sclerotherapy for an acute bleeding varix. Because the family refused consent, it was not possible to assess the efficacy or complications in this patient. The patient from Group B had an acute variceal bleed which was treated by variceal sclerotherapy. She had acute respiratory failure as well as cardiac arrest during the procedure and was successfully resuscitated. She rebled 5 days later and underwent repeat sclerotherapy. She arrested at the initiation of the procedure and died shortly thereafter. Post mortem revealed oesophageal ulceration as well as residual varices. There was almost no viable liver tissue, indicating an extremely poor prognosis. All the patients from Group B developed ulceration, 2 of which resulted in bleeding. These two patients required transfusions. They both responded well to sucralfate therapy. Three of the patients from Group B had evidence of previous stricture formation due to sclerotherapy. These strictures worsened after sclerotherapy. One of the patients from Group B developed a stricture which developed after sclerotherapy with undiluted S.T.D. Both groups of patients showed endoscopic improvement of both variceal number and variceal size after each session.

All patients were endoscoped every two weeks and sclerotherapy was continued until eradication was achieved, and thereafter the patients were followed up at four to six week intervals. The unacceptable high ulceration rate in Group B necessitated an early end to the trial.

6.4 DISCUSSION

Sclerotherapy of oesophageal varices has been associated with numerous side effects both locally and systemically. These are discussed in the next chapter.

Diluting the sclerosant has been reported previously. All these dilutions have however been empirical. Silpa et al showed in 1982 that S.T.D., when diluted to 1.5%, was as effective as 5% sodium morrhuate. Using a canine model, Jensen et al (1) evaluated various dosages and methods of administration . They showed 1.5% S.T.D. to be an effective sclerosant. They found however no difference in systemic side effects as compared with 3%. Diluting S.T.D. to 1% with normal saline has been shown to reduce the ulceration and stricture rate without significantly diminishing efficacy rate (Trudeau et al, 1983). Further dilution to 0.75% by McClave et al (1990) succeeded in reducing stricture rate although ulceration still occurred in 53% of patients. No studies so far have used a concentration as low as 0.3%. Technically, a dilution of 0.3% means a concentration of 333 fold (= 33.330%).

This very low concentration, i.e. 0.3%, is critical in that in vitro work suggested that the hypercoagulable state is not achieved at high concentrations. In fact, at high concentrations, a hypocoagulable or incoagulable state occurred. There was objective evidence of thrombosis occurring in all the patients who were injected with the dilute sclerosant.

Solution B caused an unacceptable level of ulceration supporting the concern expressed by Terblanche et al, (1990).

While accepting the obvious limitations of a pilot study viz. the relatively low numbers and poor matching in terms of the Child-Pugh classification (which was the consequence of randomisation at initial assessment), the brevity of follow-up, as well as including both patients with index bleeds together with patients on chronic sclerotherapy, there is evidence to show that the dilute sclerosant is successful for the short term management of oesophageal varices, and is not associated with unacceptable complications. The relative merit of a low dose S.T.D. induced hypercoagulable state has been addressed in the chapter on coagulation.

A larger study and longer follow up are required to determine whether this will equate with a low rebleed rate (i.e. control the varices) with fewer complications in the long term. However, to date, there have been no failures in the low dose group and the follow up of some of these patients now exceeds one year.

CHAPTER 7

7. GENERAL DISCUSSION

While sclerotherapy has regained its place as one of the modalities of choice in treating oesophageal varices, it is, as has been previously discussed, not a benign procedure. Some of the numerous complications which have been documented both locally and systemically are discussed, with particular reference to the two clinical trials.

7.1 SYSTEMIC COMPLICATIONS

One of the most disturbing systemic complications which occurs is the effect of the sclerosant on the pulmonary circulation (Monroe et al, 1983; Bacon et al, 1985; Hammond et al, 1985; De Puey et al, 1988). One of the patients in the second trial developed acute adult respiratory distress syndrome. The post mortem revealed almost no residual liver tissue, which in itself is an extremely poor prognostic indicator. However, sections of the lung contained what appeared to be sclerosant, indicating that the sclerotherapy was probably the cause of her acute respiratory failure. The findings of Chapter 2, i.e. the in vivo findings of the clinical trial on the effect of S.T.D. on the coagulation parameters, showed a transient decrease in the white cell count and fibrinogen levels. These findings are in keeping with sclerosant damage to the pulmonary circulation which resulted in the white cells being sequestrated.

One would expect that the use of a dilute sclerosant will lower the risk of damage to the pulmonary vasculature and the lungs themselves.

Haemorrhage from other sites has also been documented as a systemic complication (Foutch and Sivak, 1984; Keane et al, 1986; Fry et al,1988). In the trial in Chapter 2 the patients did not show a systemic bleeding diathesis.

Distal sites of thrombosis have been reported. (Barsoun et al, 1982; Goodale et al, 1982; Seidman et al, 1984; Ng et al, 1988). From a systemic point of view, undiluted S.T.D. as previously shown in Chapters 3 and 4, causes a local hypocoagulable state. However, marked endothelial stripping occurs simultaneously. Depending on the flow, which can be either prograde or retrograde (Gaiani et al, 1991) this high concentration will be delivered to either the pulmonary circulation or the portal venous system. Mixing of the sclerosant occurs partly as a result of the turbulent flow set up during the injection procedure and partly due to inflow of fresh blood. Dilution also occurs as a function of the distance from the site of injection. The concentration of the sclerosant will therefore diminish at the distal sites. At a low concentration S.T.D. is highly thrombogenic in that not only does it activate platelets directly and selectively inhibit Protein C, but it also strips the endothelium. This would help to explain why some patients develop portal vein thrombosis while others develop respiratory problems.

The reason why thrombosis occurs at the site of injection is that the initial high concentration of S.T.D. will decrease and "fresh" blood will supply the necessary coagulation factors and platelets, which are required for thrombus formation. A problem and a potential cause for rebleeding might be that this site of thrombus would also occur considerably downstream from the site of injection. This could potentially cause an increase in the pressure upstream from the site of thrombosis and precipitate another bleed. The fact that thrombus does occur at distal sites of the oesophageal veins was shown in 1982 by Evans et al in post mortem studies.

Cardiac complications have been described after sclerotherapy. Sodium morrhuate has been implicated in the development of bradyarrythmia (Perakos et al, 1984). However, the patient had previously documented conduction abnormalities. Acute pericarditis is an uncommon complication of sclerotherapy and a cause of chest pain (Knauer et al, 1987). Prinz Metal angina which was confirmed electrographically has also been described after sclerotherapy with ethanolamine oleate (Charng et al, 1988). Although one would intuitively believe that low dose S.T.D. would reduce incidence of these complications, this has yet to be determined.

Bacteraemia is another complication ascribed to sclerotherapy. The incidence of bacteraemia post sclerotherapy varies greatly from series to series. Camara et al (1983) reported an incidence of 5%. Cohen et al (1983) documented a much higher occurrence, 14 episodes in 11 patients. Schuman et al (1987) feel that the risk of sclerotherapy related sepsis is not associated with either the amount of the sclerosant, the difficulty of the procedure or the aetiology of the liver disease. Lange et al (1983) and Sauerbach et al (1985) recommend antibiotic prophylaxsis for patients with valvular heart disease. Schuman et al (1987) question this and feel that larger studies are required to support this approach. Although neither of the studies in this thesis addressed this question, it is doubtful whether a low dose sclerosant would alter the incidence of the bacteraemia.

Sclerotherapy with ethanolamine oleate has been associated with acute renal insufficiency (Maling et al, 1975). Miyosshi et al (1991), in a recent study, showed that renal tubular dysfunction after sclerotherapy with ethanolamine oleate could be prevented by prophylactic administration of haptoglobin. Further research is necessary to determine whether low dose S.T.D. has any effect on renal tubular function.

7.2 LOCAL COMPLICATIONS OF SCLEROTHERAPY

There have been numerous cases of local complications of sclerotherapy with varying incidences of morbidity and mortality.

7.2.1 Oesophageal Stricture

Sorenson et al (1984) demonstrated that 59% of patients developed a stricture and/or dysphagia after sclerotherapy with polidocanol. As previously stated, this is a relatively common complication occurring after sclerotherapy (Haynes et al, 1986). In the second trial (i.e. Chapter 6) none of the patients in Group A (dilute S.T.D.) developed oesophageal strictures, but 4 out of the 5 in Group B developed oesophageal strictures requiring subsequent dilatations. However in 3 of these patients, the strictures were noted to be already present at the time of initial entry into the trial. Presumably these strictures were due to previous sclerotherapy (which they were known to have had). Although it is my strong impression, as well as that of the other endoscopists involved in the trial, that these strictures worsened during this trial, it is difficult to assess the influence of the latest series of injections on this progression. The absence, however, of any stricture formation in Group A is supportive of the low complication rate subsequent upon the use of dilute sclerosant.

7.2.2 Oesophageal Perforation

Although in Perino's study 6% of patients who received sclerotherapy with 3% of S.T.D. developed perforation (Perino et al, 1987), fortunately none of the patients in either of the trials had this complication documented. One would expect that a lower dosage of S.T.D. would reduce the incidence of this potentially lethal complication.

7.2.3 Oesophageal Ulceration

The incidence of ulceration varies from series to series and would appear to be influenced by the type of sclerosant used (Sibramangam et al, 1986). Sanowski et al (1983) showed that 57% of patients who received 1.5% sodium tetradecyl sulphate developed ulceration. Ayres et al (1982) described three patients who developed ulceration with haematemesis after infection with sodium morrhuate. Two of these patients died. Westaby et al (1984) showed that 80% of patients who received sclerotherapy at weekly intervals developed ulceration as compared with 30% of patients who received sclerotherapy at three weekly intervals. The sclerosant used was ethanolamine oleate.

Sucralfate is recommended as the treatment of sclerotherapy related ulceration (Roark, 1984), although recently somatostatin has also been shown to be effective (Jenkins, 1991).

In the trial in Chapter 6, 100% of the patients in Group B (undiluted S.T.D.) developed ulcers which were still present 6 weeks after sclerotherapy. Two of these ulcers resulted in significant bleeding. These ulcers responded to sucralfate therapy. Only 1 out of 8 patients who were injected with 0.3% S.T.D. had superficial ulceration which was visualised 1 month post sclerotherapy. This responded rapidly to sucralfate therapy.

7.2.4 Chest Pain

One hundred percent of patients in the first trial (Chapter 3) complained of chest pain. The cause of this pain is thought to be due to a chemical mediastinitis but this remains controversial (Sivak et al, 1990). The incidence of chest pain was unfortunately not evaluated in the second trial. One would expect that if the mechanism is in fact due to a chemical mediastinitis then low dose sclerotherapy would reduce the incidence of this complication. This has still to be confirmed.

7.2.5 Oesophageal Dysmotility

Manometric and pH monitoring of patients with sclerotherapy has shown decreased pressure and abnormal contractions (Schuman et al, 1987). On area of future research would be to see whether low dose S.T.D. causes less dysmotility than other agents.

Other local complications which have been described include pseudotumour of the oesophagus (Korula, 1985), a mucosal bridge of the distal oesophagus (Gottfried et al, 1985), pseudodiverticula secondary to injection sclerotherapy (Scherl et al, 1983), and oesophageal monoliasis (Sarles et al, 1984). As they are rare a large series will be required to document whether low dose sclerotherapy has any effect on their incidence.

7.2.6 Carcinoma of the oesophagus

Bochna et al (1988) reported a case of squamous cell carcinoma of the oesophagus. This occurred 8 months after sclerotherapy with sodium morrhuate. Due to the rarity of this complication it is difficult to prove a causal relationship between sclerotherapy and carcinoma.

7.3 CONCLUSIONS

Sclerotherapy of oesophageal varices is undoubtedly an extremely useful weapon in the armamentarium for treating oesophageal varices. Most of the current methodology employed is based on empirical approaches.

The object of the thesis was to evaluate the effect of sodium tetradecyl sulphate on endothelium, the coagulation pathway and platelets. The central recurring theme of this thesis has been the documentation of a thrombogenic effect of sclerotherapy, occurring at concentrations of S.T.D. ten fold less than those actually injected, following current practice. Thus, localised endothelial stripping, hypercoagulability and platelet aggregation were all documented following exposure to low concentrations of S.T.D. In contrast, exposure to high concentrations of S.T.D. led to more widespread endothelial stripping, blood incoagulability and platelet lysis. These findings suggest that a low concentration of S.T.D. may be at least as effective as standard concentration S.T.D. in the management of acute bleeding varices and possibly more so, and would have the added benefit of limiting most of the associated complications. A clinical pilot study based on these findings supported this view.

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APPENDIX

FACTOR XII (% OF CONTROL)

STD	1	2	3	4	5	6	7
NEAT	100	100	100	100	100	100	100
10µl	102	65	106	100	89	88	106
25µl	100	106	98	85	100	87	112
35µl	119	120	-	98	102	88	102
50µl	98	103	95	109	103	89	99
100µl	101	104	81	96	90	87	104
150µl	103	82	72	80	93	68	92
200µl	83	68	63	86	69	60	75
350µl	-	-	48	68	49	48	53

DATA FROM FIGURE 4.4

FACTOR XII (% OF CONTROL)

X VALUE	Y MEAN	Y TOTAL	Y MINIMUM
NEAT	100	700	100
10 µl	93.7143	656	65
25 µl	98.2857	688	85
35 µl	103.3333	620	88
50 µl	99.4286	696	89
100 µl	94.7143	663	81
150 µl	84.2857	590	68
200 µl	72	504	60
350 µl	53.2	266	48

X VALUE	Y MAX	SD (n-1)	SD (n)	SEM	N
NEAT	100	0	0	0	7
10 µl	106	14.6596	13.8722	5.5408	7
25 µl	112	9.6387	8.9237	3.6431	7
35 µl	120	10.8566	9.9107	4.4322	6
50 µl	109	6.4254	5.9488	2.4286	7
100 µl	104	8.9762	8.3103	3.3927	7
150 µl	103	12.4193	11.4980	4.6940	7
200 µl	86	9.7980	9.0711	3.7033	7
350 µl	68	8.5264	7.6263	3.8131	5

DATA FROM FIGURE 4.4

FACTOR XII (% OF CONTROL)

N = 9	MEAN	TOTAL	MINIMUM
X-DATA	102.2222	920	0
Y-DATA	88.7735	798.9619	53.2

N = 9	MAXIMUM	SD (N-1)	SD (N)	SEM
X-DATA	350	114.7854	108.2207	38.2618
Y-DATA	103.3333	16.4648	15.5231	5.4883

LINEAR REGRESSION OF Y ON X

Slope of line	=	-0.138086
Y-Intercept	=	102.8890
X-Intercept	=	745.1098
XY Correlation	=	-0.962676
(Correlation)2	=	0.926745

AREA UNDER THE CURVE

Trapezoidal	=	27563.09
Start-Rectangular	=	29660
End-Rectangular	=	25466.19

DATA FROM FIGURE 4.4

FACTOR XI (% OF CONTROL)

STD	1	2	3	4	5	6	7
NEAT	100	100	100	100	100	100	100
10µl	100	94	105	94	92	93	105
25µl	99	98	97	94	102	96	94
35µl	107	98	-	94	97	94	94
50µl	103	97	94	109	101	91	96
100µl	114	93	81	93	104	91	102
150µl	115	78	68	87	108	73	87
200µl	108	59	67	79	94	70	82
350µl	-	-	46	44	70	49	53

DATA FROM FIGURE 4.5

FACTOR XI (% OF CONTROL)

X VALUE	Y MEAN	Y TOTAL	Y MINIMUM
NEAT	100	700	100
10µl	97.5714	683	92
25µl	97.1429	680	94
35µl	97.3333	584	94
50µl	98.7143	691	91
100µl	96.8571	678	81
150µl	88	616	68
200µl	79.8571	559	59
350µl	52.400	262	44

X VALUE	Y MAX	SD (n-1)	SD (n)	SEM	NO
NEAT	100	0	0	0	7
10µl	105	5.6821	5.2606	2.1476	7
25µl	102	2.8536	2.6419	1.0785	7
35µl	107	5.0465	4.6068	2.0602	6
50µl	109	6.0749	5.6243	2.2961	7
100µl	114	10.6994	9.9057	4.0440	7
150µl	115	17.5879	16.2832	6.6476	7
200µl	108	16.8070	15.5603	6.3525	7
350µl	70	10.4067	9.3081	4.6540	5

DATA FROM FIGURE 4.5

FACTOR XI (% OF CONTROL)

N = 9	MEAN	TOTAL	MINIMUM
X-DATA	102.2222	920	0
Y-DATA	89.7540	807.8762	52.4

N = 9	MAXIMUM	SD (N-1)	SD (N)	SEM
X-DATA	350	114.7854	108.2207	38.2618
Y-DATA	100	15.4322	14.5496	5.1441

LINEAR REGRESSION OF Y ON X

Slope of line	=	-0.129396
Y-Intercept	=	102.9912
X-Intercept	=	795.9361
XY Correlation	=	-0.962456
(Correlation)2	=	0.926322

AREA UNDER THE CURVE

Trapezoidal	=	28517.38
Start-Rectangular	=	31052.14
End-Rectangular	=	25982.61

DATA FROM FIGURE 4.5

FACTOR X (% OF CONTROL)

STD	1	3	3	4	5	6
NEAT	100	100	100	100	100	100
10µl	92	95	100	96	103	107
25µl	92	91	100	96	101	100
35µl	88	-	94	91	89	100
50µl	88	84	103	89	90	98
100µl	88	77	94	89	84	100
150µl	82	71	94	82	86	83
200µl	74	68	79	72	75	80
350µl	-	17	14	32	20	13

DATA FROM FIGURE 4.6

FACTOR X (% OF CONTROL)

X VALUE	Y MEAN	Y TOTAL	Y MINIMUM
NEAT	100	600	100
10µl	98.8333	593	92
25µl	96.6667	580	91
35µl	92.4000	462	88
50µl	92	552	84
100µl	88.6667	532	77
150µl	83	498	71
200µl	74.6667	448	68
350µl	19.2000	96	13

X VALUE	Y MAX	SD (n-1)	SD (n)	SEM	n
NEAT	100	0	0	0	6
10µl	107	5.5648	5.0799	2.2718	6
25µl	101	4.3665	3.9861	1.7826	6
35µl	100	4.8270	4.3174	2.1587	5
50µl	103	7.0711	6.4550	2.8868	6
100µl	100	7.9415	7.2495	3.2421	6
150µl	94	7.4297	6.7823	3.0332	6
200µl	80	4.4572	4.0689	1.8196	6
350µl	32	7.6616	6.8527	3.4264	5

DATA FROM FIGURE 4.6

FACTOR X (% OF CONTROL)

N = 9	MEAN	TOTAL	MINIMUM
X-DATA	102.2222	920	0
Y-DATA	82.8259	745.4333	19.2

N = 9	MAXIMUM	SD (n-1)	SD (n)	SEM
X-DATA	350	114.7854	108.3207	38.2618
Y-DATA	100	25.1683	23.7289	8.3894

LINEAR REGRESSION OF Y ON X

Slope of line	=	-0.208631
Y-Intercept	=	104.1526
X-Intercept	=	499.2197
XY Correlation	=	-0.951505
(Correlation)2	=	0.905362

AREA UNDER THE CURVE

Trapezoidal	=	24578.75
Start-Rectangular	=	29218.50
End-Rectangular	=	19939

DATA FROM FIGURE 4.6

FACTOR IX (% OF CONTROL)

STD	1	2	3	4	5	6
NEAT	100	100	100	100	100	100
10µl	95	98	94	92	88	101
25µl	108	91	94	110	95	110
35µl	92	-	94	100	91	101
50µl	113	90	108	108	96	104
100µl	103	79	93	99	88	104
150µl	86	71	87	102	80	91
200µl	58	66	79	91	70	66
350µl	-	39	44	53	44	32

DATA FROM FIGURE 4.7

FACTOR IX (% OF CONTROL)

X VALUE	Y MEAN	Y TOTAL	Y MINIMUM
NEAT	100	600	100
10µl	94.6667	568	88
25µul	101.3333	608	91
35µl	95.600	478	91
50µl	103.1667	619	90
100µl	94.3333	566	79
150µl	86.1667	517	71
200µl	70	420	58
350µl	42.400	212	32

X VALUE	Y MAX	SD (n-1)	SD (n)	SEM	n
NEAT	100	0	0	0	6
10µl	101	4.5461	4.1500	1.8559	6
25µl	110	8.8919	8.1172	3.6301	6
35µl	101	4.6152	4.1280	2.0640	5
50µl	113	8.5888	7.8404	3.5063	6
100µl	104	9.6678	8.8255	3.9469	6
150µl	102	10.4195	9.5117	4.2538	6
200µl	81	8.6948	7.9373	3.5496	6
350µl	53	7.7006	6.8877	3.4438	5

DATA FROM FIGURE 4.7

FACTOR IX (% OF CONTROL)

N = 9	MEAN	TOTAL	MINIMUM
X-DATA	102.2222	920.000	0.0000
Y-DATA	87.5185	787.6667	42.4000

N = 9	MAXIMUM	SD (N-1)	SD (N)	SEM
X-DATA	350	114.7854	108.2207	38.2613
Y-DATA	103.1667	19.6811	18.5555	6.5634

LINEAR REGRESSION OF Y ON X

Slope of line	=	-0.164454
Y-Intercept	=	104.3294
X-Intercept	=	634.3983
XY Correlation	=	-0.959141
(Correlation)2	=	0.919952

AREA UNDER THE CURVE

Trapezoidal	=	26702.91
Start-Rectangular	=	29550.66
End-Rectangular	=	23855.16

DATA FROM FIGURE 4.7

FACTOR VIII (% OF CONTROL)

STD	1	2	3	4	5	6	7
NEAT	100	100	100	100	100	100	100
10µl	108	106	98	101	95	89	9[illegible]
25µl	90	100	92	104	101	95	93
35µl	121	103	[illegible]	94	100	87	88
50µl	104	97	86	92	104	90	91
100µl	107	89	78	88	95	80	93
150µl	72	76	66	72	98	62	71
200µl	50	46	59	62	76	58	65
350µl	-	-	38	38	49	42	49

DATA FROM FIGURE 4.8

FACTOR VIII (% OF CONTROL)

X VALUE	Y MEAN	Y TOTAL	Y MINIMUM
NEAT	100	700	100
10µL	99.1429	694	89
25µl	96.4286	675	90
35µl	98.8333	593	87
50µl	94.8571	664	86
100µl	90	630	78
150µl	73.8571	517	62
200µl	59.4286	416	46
350µl	43.200	216	38

X VALUE	Y MAX	SD (n-1)	SD (n)	SEM	n
NEAT	100	0	0	0	7
10µl	108	6.5174	6.0339	2.4633	7
25µl	104	5.2554	4.8655	1.9863	7
35µl	121	12.5764	11.4807	5.1343	6
50µl	104	7.0339	6.5122	2.6586	7
100µl	107	9.7639	9.0396	3.6904	7
150µl	98	11.5820	10.7229	4.3776	7
200µl	76	9.8634	9.1317	3.7280	7
350µl	49	5.5408	4.9558	2.4779	5

DATA FROM FIGURE 4.8

FACTOR VIII (% OF CONTROL)

X VALUE	Y MEAN	Y TOTAL	Y MINIMUM
NEAT	100	700	100
10µL	99.1429	694	89
25µl	96.4286	675	90
35µl	98.8333	593	87
50µl	94.8571	664	86
100µl	90	630	78
150µl	73.8571	517	62
200µl	59.4286	416	46
350µl	43.200	216	38

X VALUE	Y MAX	SD ($n-^1$)	SD (n)	SEM	n
NEAT	100	0	0	0	7
10µl	108	6.5174	6.0339	2.4633	7
25µl	104	5.2554	4.8655	1.9863	7
35µl	121	12.5764	11.4807	5.1343	6
50µl	104	7.0339	6.5122	2.6586	7
100µl	107	9.7639	9.0396	3.6904	7
150µl	98	11.5820	10.7229	4.3776	7
200µl	76	9.8634	9.1317	3.7280	7
350µl	49	5.5408	4.9558	2.4779	5

DATA FROM FIGURE 4.8

FACTOR VIII (% OF CONTROL)

N = 9	MEAN	TOTAL	MINIMUM
X-DATA	102.2222	920	0
Y-DATA	83.9720	755.7476	43.20

N = 9	MAXIMUM	SD (N-1)	SD (N)	SEM
X-DATA	350	114.7854	108.2207	38.2618
Y-DATA	100	20.5698	19.3934	6.8566

LINEAR REGRESSION OF Y ON X

Slope of line	=	-0.176136
Y-Intercept	=	101.9770
X-Intercept	=	578.9663
XY Correlation	=	-0.982890
(Correlation)2	=	0.966072

AREA UNDER THE CURVE

Trapezoidal	=	24638.63
Start-Rectangular	=	26783.92
End-Rectangular	=	22493.33

DATA FROM FIGURE 4.8

FACTOR VII (% OF CONTROL)

STD	1	2	3	4	5	6
NEAT	100	100	100	100	100	100
10µl	91	93	100	89	100	98
25µl	90	88	102	91	98	97
35µl	93	-	93	87	88	97
50µl	94	83	96	83	90	92
100µl	91	72	96	80	79	92
150µl	82	67	94	72	83	85
200µl	71	64	71	60	72	62
350µl	-	31	35	25	38	18

DATA FROM FIGURE 4.9

FACTOR VII (% OF CONTROL)

X VALUE	Y MEAN	Y TOTAL	Y MINIMUM
NEAT	100	600	100
10µl	95.1667	571	89
25µl	94.3333	566	88
35µl	91.6000	458	87
50µl	89.6667	538	83
100µl	85	510	72
150µl	80.5	483	67
200µl	66.6667	400	60
350µl	29.4	147	18

X VALUE	Y MAX	SD (n-1)	SD (n)	SEM	n
NEAT	100	0	0	0	6
10µl	100	4.7924	4.3748	1.9565	6
25µl	102	5.4650	4.9889	2.2311	6
35µl	97	4.0988	3.6661	1.8330	5
50µl	96	5.5377	5.0553	2.2608	6
100µl	96	9.3381	8.5245	3.8123	6
150µl	94	9.6488	8.8081	3.9391	6
200µl	72	5.2789	4.8189	2.1551	6
350µl	38	8.0187	7.1722	3.5861	5

DATA FROM FIGURE 4.9

FACTOR VII (% OF CONTROL)

N = 9	MEAN	TOTAL	MINIMUM
X-DATA	102.2222	920	0
Y-DATA	81.3704	732.3333	29.400

	MAXIMUM	SD (N-1)	SD (N)	SEM
X-DATA	350	114.7854	108.2207	38.2618
Y-DATA	100	21.8230	20.5750	7.2743

LINEAR REGRESSION OF Y ON X

Slope of line	=	-0.186547
Y-Intercept	=	100.4396
X-Intercept	=	538.4149
XY Correlation	=	-0.981204
(Correlation) 2	=	0.962761

AREA UNDER THE CURVE

Trapezoidal	=	24074.58
Start-Rectangular	=	27503.16
End-Rectangular	=	20646.00

DATA FROM FIGURE 4.9

FACTOR V (% OF CONTROL)

STD	1	2	3	4	5	6	7
NEAT	100	100	100	100	100	100	100
10µl	98	89	98	114	98	107	104
25µl	92	91	83	114	98	98	99
35µl	109	91	-	106	95	87	99
50µl	106	91	88	117	88	91	100
100µl	118	95	80	118	91	82	109
150µl	118	83	72	97	81	87	98
200µl	69	44	51	54	53	48	53
350µl	-	-	21	26	21	17	24

DATA FROM FIGURE 4.10

FACTOR V (% OF CONTROL)

X VALUE	Y MEAN	Y TOTAL	Y MINIMUM
NEAT	100	700	100
10µl	101.1429	708	89
25µl	96.4286	675	83
35µl	97.8333	587	87
50µl	97.2857	681	88
100µl	99	693	80
150µl	90.8571	636	72
200µl	53.1429	372	44
350µl	21.8000	109	17

X VALUE	Y MAX	SN (n-1)	SD (n)	SEM	NO
NEAT	100	0	0	0	7
10µl	114	8.0039	7.4148	3.0271	7
25µl	114	9.5718	8.8617	3.6178	7
35µl	109	8.5421	7.7978	3.4873	6
50µl	117	10.9805	10.1660	4.1502	7
100µl	118	16.0831	14.8901	6.0788	7
150µl	118	15.0270	13.9123	5.6797	7
200µl	69	7.8194	7.2393	2 9555	7
350µl	26	3.4205	3.0594	1.5297	5

DATA FROM FIGURE 4.10

FACTOR V (% OF CONTROL)

N = 9	MEAN	TOTAL	MINIMUM
X-DATA	102.2222	920	0
Y-DATA	84.1656	757.4905	21.8

N = 9	MAXIMUM	SD (N-1)	SD (N)	SEM
X-DATA	350	114.7854	108.2207	38.2618
Y-DATA	101.1429	27.7601	26.1725	9.2534

LINEAR REGRESSION OF Y ON X

Slope of line	=	-0.227072
Y-Intercept	=	107.3774
X-Intercept	=	472.8787
XY Correlation	=	-0.938920
(Correlation)2	=	0.881570

AREA UNDER THE CURVE

Trapezoidal	=	23796.48
Start-Rectangular	=	27277.50
End-Rectangular	=	20315.47

DATA FROM FIGURE 4.10

FACTOR II (% OF CONTROL)

STD	1	2	3	4	5	6	7
NEAT	100	100	100	100	100	100	100
10µl	100	99	98	102	100	100	105
25µl	101	96	94	107	100	105	102
35µl	102	95	-	97	98	87	102
50µl	98	98	89	102	89	95	84
100µl	96	98	79	97	89	89	92
150µl	95	92	73	102	80	92	82
200µl	83	80	74	83	75	79	45
350µl	-	-	42	44	44	41	20

DATA FROM FIGURE 4.11

FACTOR II (% OF CONTROL)

X VALUE	Y MEAN	Y TOTAL	Y MINIMUM
NEAT	100	700	100
10µl	100.5714	704	98
25µl	99.8571	699	90
35µl	96.8333	581	87
50µl	93.5714	655	84
100µl	91.4286	640	79
150µl	88	616	73
200µl	74.1429	519	45
350µl	38.2000	191	20

X VALUE	Y MAX	SD (n-1)	SD (n)	SEM	n
NEAT	100	0	0	0	7
10µl	105	2.2991	2.1285	0.868966	7
25µl	107	5.9841	5.5402	2.2618	7
35µl	102	5.5648	5.0799	2.2718	6
50µl	102	6.3994	5.9247	2.4187	7
100µl	98	6.6045	6.1146	2.4963	7
150µl	102	10.0167	9.2736	3.7859	7
200µl	83	13.3220	12.3338	5.0352	7
350µl	44	10.2567	9.1739	4.5869	5

DATA FROM FIGURE 4.11

FACTOR II (% OF CONTROL)

N = 9	MEAN	TOTAL	MINIMUM
X-DATA	102.2222	920	0
Y-DATA	86.9561	782.6048	38.2000

N = 9	MAXIMUM	SD (n-1)	SD (n)	SEM
X-DATA	350	114.7854	108.2207	38.2618
Y-DATA	100.5714	20.0802	18.9318	6.6934

LINEAR REGRESSION OF Y ON X

Slope of line	=	-0.169709
Y-Intercept	=	104.3041
X-Intercept	=	614.6052
XY Correlation	=	-0.970118
(Correlation) 2	=	0.941128

AREA UNDER THE CURVE

Trapezoidal	=	26507.55
Start-Rectangular	=	29731.07
End-Rectangular	=	23284.04

DATA FROM FIGURE 4.11

FIBRINOGEN (% OF CONTROL)

STD	1	2	3	4	5	6
NEAT	100	100	100	100	100	100
10µl	96	93	104	86	102	73
25µl	94	95	98	90	100	76
35µl	98	-	93	82	101	70
50µl	89	92	106	80	99	69
100µl	91	81	97	76	91	68
150µl	85	77	96	67	86	60
200µl	0	70	89	66	90	68
350µl	-	0	0	51	76	65

DATA FROM FIGURE 4.12

FIBRINOGEN (% OF CONTROL)

X VALUE	Y MEAN	Y TOTAL	Y MINIMUM
NEAT	100	600	100
10µl	92.3333	554	73
25µl	92.1667	553	76
35µl	88.8	444	70
50µl	89.1667	535	69
100µl	84	504	68
150µl	78.5	471	60
200µl	63.8333	383	0
350µl	38.4	192	0

X VALUE	Y MAX	SD (n-1)	SD (n)	SEM	n
NEAT	100	0	0	0	6
10µl	104	11.4659	10.4669	4.6809	6
25µl	100	8.6352	7.8828	3.5253	6
35µl	101	12.7554	11.4088	5.7044	5
50µl	106	13.2577	12.1026	5.4124	6
100µl	97	10.9179	9.9666	4.4572	6
150µl	96	13.2778	12.1209	5.4206	6
200µl	90	33.0237	30.1464	13.4819	6
300µl	76	36.1566	32.3394	16.1697	5

DATA FROM FIGURE 4.12

FIBRINOGEN (% OF CONTROL)

N = 9	MEAN	TOTAL	MINIMUM
X-DATA	102.2222	920	0
Y-DATA	80.8000	727.2000	38.4

N = 9	MAXIMUM	SD (N-1)	SD (N)	SEM
X-DATA	350	114.7854	108.2207	38.2618
Y-DATA	100	18.9276	17.8451	6.3092

LINEAR REGRESSION OF Y ON X

Slope of line	=	-0.162805
Y-Intercept	=	97.4422
X-Intercept	=	598.5230
XY Correlation	=	-0.987322
(Correlation)2	=	0.974804

AREA UNDER THE CURVE

Trapezoidal	=	24202.50
Start-Rectangular	=	26797
End-Rectangular	=	21608

DATA FROM FIGURE 4.12

PROTEIN C (% OF CONTROL)

STD	1	2	3	4	5	6	7
NEAT	-	100	100	100	100	100	100
10µl	-	116	88	84	72	98	84
25µl	-	82	95	60	66	73	63
35µl	-	103	-	67	63	68	69
50µul	-	87	98	50	72	60	66
100µl	-	86	78	70	71	64	63
150µl	-	79	98	64	60	66	57
200µl	-	50	79	60	75	61	43
350µl	-	-	28	27	45	33	19

DATA FROM FIGURE 4.13

PROTEIN C (% OF CONTROL)

X VALUE	Y MEAN	Y TOTAL	Y MINIMUM
NEAT	100	600	100
10µul	90.333	542	72
25µul	73.1 7	439	60
35µul	74	370	63
50µul	72.1667	433	50
100µul	72	432	63
150µl	70.6667	424	57
200µul	61.333	368	43
350µl	30.400	152	19

X VALUE	Y MAX	SD (n-1)	SD (n)	SEM	NO
NEAT	100	0	0	0	6
10µl	116	15.0953	13.7800	6.1626	6
25µl	98	13.2878	12.1301	5.4247	6
35µl	103	16.3707	14.6424	7.3212	5
50µl	98	17.6909	16.1495	7.2223	6
100µl	86	8.7407	7.9791	3.5684	6
150µl	98	15.3840	14.0436	6.2805	6
200µl	79	13.8948	12.6842	5.6725	6
350µl	45	9.5812	8.5697	4.2849	5

DATA FROM FIGURE 4.13

PROTEIN C (% OF CONTROL)

N = 9	MEAN	TOTAL	MINIMUM
X-DATA	102.2222	920	0
Y-DATA	71.5630	644.0667	30.4

N = 9	MAXIMUM	SD (N-1)	SD (N)	SEM
X-DATA	350	114.7854	108.2207	38.2618
Y-DATA	100	19.2675	18.1656	6.4225

LINEAR REGRESSION OF Y ON X

Slope of Line	=	-0.153696
Y-Intercept	=	87.2741
X-Intercept	=	567.8355
XY Correlation	=	-0.915638
(Correlation)2	=	0.838394

AREA UNDER THE CURVE

Trapezoidal	=	21360.83
Start-Rectangular	=	24138.33
End-Rectangular	=	18583.33

DATA FROM FIGURE 4.13

PTT

STD	1	2	3	4	5	6	7
NEAT	36.5	33.1	34	33.5	37.4	33	35.6
10 µl	37.5	30.4	36.7	40	34.3	32.6	34.5
25 µl	39.1	30.7	36.1	35.5	33.5	34	32.8
35 µl	43	31.9	-	36.8	37.6	34.9	33.2
50 µl	42	33.8	36	37.5	34.2	34.3	34.9
100µl	32.5	39.2	37.5	46	37.2	39.8	36
150µl	42	46	46	57	41.2	45	45.9
200µl	61.1	92.9	73	91.5	58.1	63.4	70.5
350µl	-	-	120	120	120	120	120

DATA FROM FIGURE 4.14

PTT

X VALUE	Y MEAN	Y TOTAL	Y MINIMUM
NEAT	34.7286	243.1	33
10 µl	35.1429	246	30.4
25 µl	34.5286	241.7	30.7
35 µl	36.2333	217.4	31.9
50 µl	36.1	252.7	33.8
100 µl	38.3143	268.2	32.8
150 µl	46.1571	323.1	41.2
200 µl	72.9286	510.5	58.1
350 µl	120	600	120

X VALUE	Y MAX	SD (n-1)	SD (n)	SEM	n
NEAT	37.4	1.7661	1.6351	0.667516	7
10 µl	40	3.2056	2.9679	1.2116	7
25 µl	39.1	2.6862	2.4869	1.0153	7
35 µl	43	3.9429	3.5994	1.6097	6
50 µl	42	2.6971	2.6822	1.0950	7
100 µl	46	4.1459	3.8383	1.5670	7
150 µl	57	5.1781	4.7940	1.9571	7
200 µl	92.9	14.1436	13.0945	5.3458	7
350 µl	120	0	0	0	5

DATA FROM FIGURE 4.14

PTT

N = 9	MEAN	TOTAL	MINIMUM
X-DATA	102.2222	920	0
Y-DATA	50.4593	454.1333	34.5286

N = 9	MAXIMUM	SD (N-1)	SD (N)	SEM
X-DATA	350	114.7854	108.2207	38.2618
Y-DATA	120	28.8340	37.1849	9.6113

LINEAR REGRESSION OF Y ON X

Slope of line	=	0.239134
Y-Intercept	=	26.0145
X-Intercept	=	-108.7865
XY Correlation	=	0.951968
(Correlation)2	=	0.906244

AREA UNDER CURVE

Trapezoidal	=	23187.13
Start-Rectangular	=	18731.07
End-Rectangular	=	27643.19

DATA FROM FIGURE 4.14

PTT

N = 9	MEAN	TOTAL	MINIMUM
X-DATA	102.2222	920	0
Y-DATA	50.4593	454.1333	34.5286

N = 9	MAXIMUM	SD (N-1)	SD (N)	SEM
X-DATA	350	114.7854	108.2207	38.2618
Y-DATA	120	28.8340	27.1849	9.6113

LINEAR REGRESSION OF Y ON X

Slope of line	=	0.239134
Y-Intercept	=	26.0145
X-Intercept	=	-108.7865
XY Correlation	=	0.951968
(Correlation)2	=	0.906244

AREA UNDER CURVE

Trapezoidal	=	23187.13
Start-Rectangular	=	18731.07
End-Rectangular	=	27643.19

DATA FROM FIGURE 4.14

INR

STD	1	2	3	4	5	6	7
NEAT	1.05	1.25	1.06	1.09	1.02	1.04	1.1
10 µl	1.07	1.53	1.07	1.12	1	1.05	1.08
25 µl	1.21	1.46	1.16	1.09	1.05	1.06	1.11
35 µl	1.08	1.31	-	1.12	1.09	1.08	1.11
50 µl	1.15	1.56	1.19	1.15	1	1.16	1.1
100µl	1.12	1.47	1.11	1.12	1.06	1.19	1.12
150µl	1.23	1.47	1.23	1.18	1.31	1.18	1.17
200µl	1.37	2.63	1.52	1.39	1.49	1.53	1.5
350µl	-	-	4.64	4.52	10	10	3.68

DATA FROM FIGURE 4.15

INR

X VALUE	Y MEAN	Y TOTAL	Y MINIMUM
NEAT	1.0871	7.61	1.02
10 µl	1.1314	7.92	1
25 µl	1.1629	8.14	1.05
35 µl	1.1317	6.79	1.08
50 µl	1.1886	8.32	1
100 µl	1.17	8.19	1.06
150 µl	1.2529	8.77	1.17
200 µl	1.6329	11.43	1.37
350 µl	6.5680	32.84	3.68

X VALUE	Y MAX	SD (n-1)	SD (n)	SEM	n
NEAT	1.25	0.076966	0.071257	0.029091	7
10 µl	1.53	0.179391	0.166083	0.067803	7
25 µl	1.46	0.142562	0.131986	0.053883	7
35 µl	1.31	0.088863	0.081121	0.036278	6
50 µl	1.56	0.175350	0.162343	0.066276	7
100 µl	1.47	0.137598	0.127391	0.052007	7
150 µl	1.47	0.107194	0.099242	0.040515	7
200 µl	2.63	0.444174	0.411225	0.167882	7
350 µl	10	3.1547	2.8217	1.4108	5

DATA FROM FIGURE 4.15

INR

N = 9	MEAN	TOTAL	MINIMUM
X-DATA	102.2222	920	0
Y-DATA	1.8139	16.3254	1.0871

N = 9	MAXIMUM	SD (n-1)	SD (n)	SEM
X-DATA	350	114.7854	108.2207	38.2618
Y-DATA	6.5680	1.7902	1.6878	0.596726

LINEAR REGRESSION OF Y ON X

Slope of line	=	0.013291
Y-Intercept	=	0.455334
X-Intercept	=	-34.2598
XY Correlation	=	0.852189
(Correlation)2	=	0.726226

AREA UNDER THE CURVE

Trapezoidal	=	863.9173
Start-Rectangular	=	481.9464
End-Rectangular	=	1245.888

DATA FROM FIGURE 4.15

TABLE OF TEG VALUES

r Value

	1		2		3	
	i	ii	i	ii	i	ii
NEAT	13		11	12	10.5	12
25 µl	10	10	9	9.5	8.5	10.5
30 µl	8	7	7.5	8.5	7	8
35 µl	12.5	12	10	11.5	9.5	8
40 µl	13	12	1	12.5	11	10.5
45 µl	21.5	20.5	23	21.5	19.5	2

k Value

	1		2		3	
	i	ii	i	ii	i	ii
NEAT	6	5	4.5	5.5	6	5
25 µl	3	3	3.5	3	4	3
30 µl	3	3	3.5	3	4	3

Maximal Amplitude

	1		2		3	
	i	ii	i	ii	i	ii
NEAT	45	48	52	51	44	40
25 µl	23	26	35	38	28	24
30 µl	21	25	30	29	25	20
35 µl	10	12	14	11	10.5	12
40 µl	11	12	14	10	9	10
45 µl	6	8	9	6	5	6