Effects of Puff-Adder Venom on Coagulation, Fibrinolysis and Platelet Aggregation in the Baboon

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SUMMARY

The *in vitro* and *in vivo* haematological effects of puff-adder (*Bitis arietans*) venom in the baboon (*Papio ursinus*) with regard to its effect on coagulation, fibrinolysis and platelet aggregation were studied. There is a delay in the intrinsic coagulation mechanism with fibrinolysis and *in vitro* fibrinogenolysis. Normal human platelets demonstrated an extreme susceptibility to puff-adder venom *in vitro*. The aggregation of platelets with small dosages of venom was irreversible.

The effect is related to the dosage, and appears to be the result of multiple enzyme activities, some of which are heat-labile. In minimal amounts there is evidence of consumption of blood coagulation factors with sustained thrombocytopenia, but no fibrinolysis, where heparin therapy might have a beneficial effect. With larger dosages of venom the thrombocytopenia, haemorrhagic effects and shock are not prevented or corrected by heparin and heparinisation might even be harmful.

The effect of heparin should be further investigated, since it might have a place in mildly affected cases of snakebite, where absorption into the blood has been slow or intermittent. It might be indicated when antivenene is not available or when patients are sensitive to serum.

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The puff-adder has been shown to be responsible for most fatal snakebites in the Cape Province.[1] It belongs to the erectile-fanged group of snakes, and its fangs are relatively long (10 - 15 mm), recurved and needle-sharp, with the orifice for the discharge of the venom near the tip; they are mounted right at the front of the upper jaw and can be rotated through 90° to ensure that the venom is injected sufficiently deep into the tissues.[2]

Recent reports indicate that disseminated intravascular coagulopathy (DIC) may be the underlying mode of action of many snakes' venoms.[3] Puff-adder venom has not been investigated in this respect. This is a preliminary report on the *in vitro* and *in vivo* effects of puff-adder venom on coagulation, fibrinolysis and platelet aggregation in the baboon. Special attention was given to the use of heparin as part of the treatment.

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MATERIALS AND METHODS

Venom

Lyophilised *Bitis arietans* venom was obtained from Professor G. R. Delpiérre, Department of Chemistry, University College of the Western Cape. The venom was reconstituted in 0,9% sterile saline just before use.

Baboons

Unconditioned male or female baboons weighing 10 - 15 kg were used. They were sedated with Sernylan 12 mg initially, and lightly anaesthetised with intravenous Veterinary Nembutal during the course of the experiments. Cut-downs on both femoral arteries were done and T-tubes inserted; one connected to a manometer measuring the blood pressure and pulse rate, and the other used for taking arterial blood samples. A slow intravenous saline drip was used for injecting Nembutal and various concentrations of venom. An indwelling catheter was placed in the bladder and the output of urine measured.

Laboratory Studies

Standard techniques were used. Full blood counts were done with the Coulter Model S, and platelet counts were done with the Coulter Model F. Blood smears were stained with May-Grünewald-Giemsa. Clottable plasma fibrinogen concentrations were measured by a modification of Ratnoff and Menzie's method.[4] Partial thromboplastin time (PTT), prothrombin index (PI) and thrombin time were done according to the method of Dacie and Lewis.[5] Prothrombin activity (PA) was done according to Owren's method.[6] Tanned red cell haemagglutination inhibition immunoassay (TRCHIIA) was done by the method of Merskey *et al.*[7] Euglobulin lysis time was done as described by Thompson.[8] The fibrin plate lysis method used was slightly modified from Ygge *et al.*[9] The platelet aggregation studies were done as described by Thompson.[8]

RESULTS

Incubation of Venom with Purified Fibrinogen

This was carried out as described by MacKay *et al.*[10] (Fig. 1).

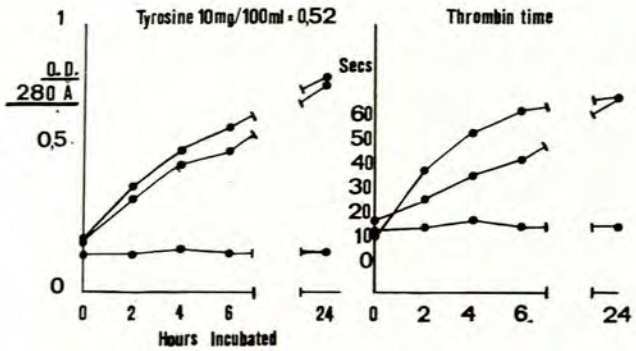

Fig. 1. *In vitro* **incubation of venom with purified fibrinogen—see text.** Upper graphs: **Undenatured venom and fibrinogen.** Middle graphs: **Heat denatured venom and fibrinogen.** Lower graphs: **Control of saline and fibrinogen.**

After protein precipitation the supernatant demonstrated a progressive increase of optical density at 280 Å and prolonged thrombin time, with complete incoagulability of the test after 24 hours. Two fractions of venom were noticed with heat-stable fibrinogenolytic effects *in vitro*.

Coagulation Studies in vitro

Clottable fibrinogen PTT, PI and PA were performed in the presence of varying concentrations of venom with two control tests without venom (Fig.2).

Large doses of venom: The most striking effect was on the clottable fibrinogen concentration and the PTT; the plasma becoming incoagulable in these two tests in the presence of large doses of venom. The PI and PA were slightly prolonged. The intrinsic coagulation mechanism is thus particularly susceptible to venom. The last column in

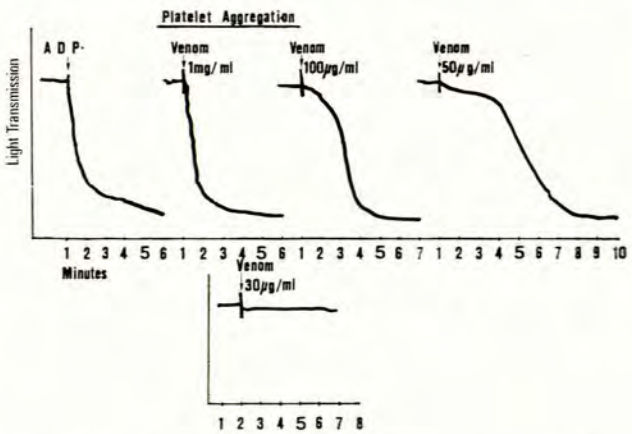

Fig. 3. Platelet aggregation studies *in vitro*—**see text. The effect of ADP upon platelet aggregation served as a control.**

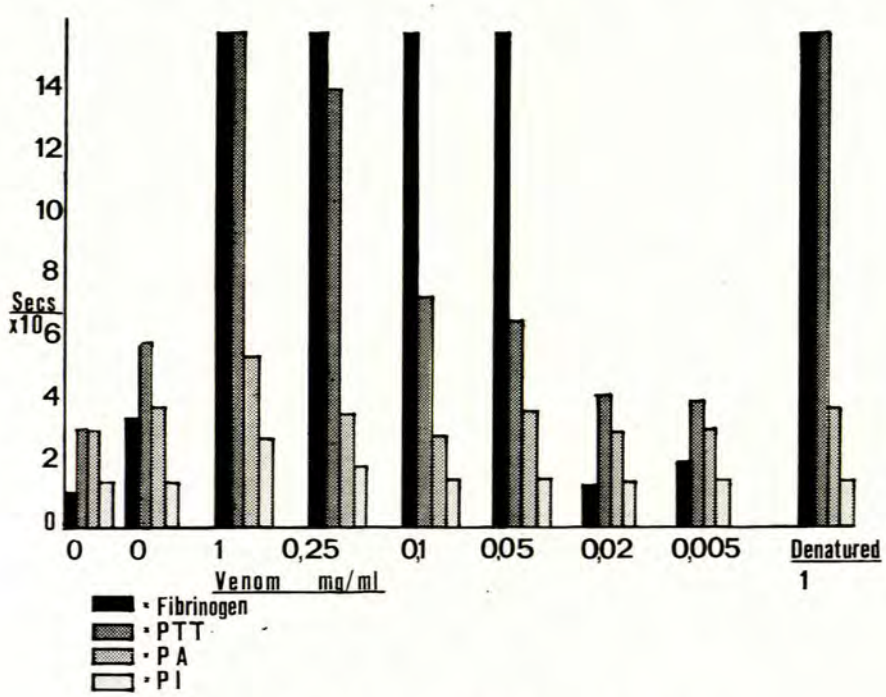

Fig. 2. Coagulation studies *in vitro*—**see text.**

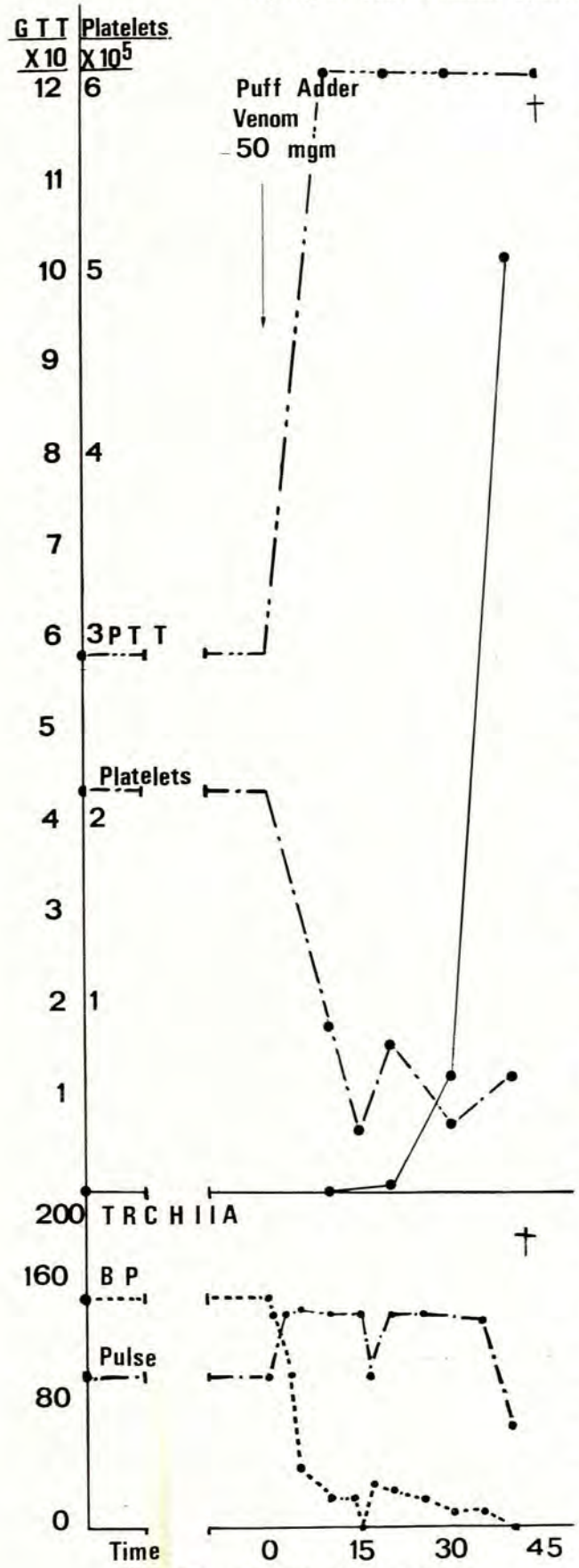

Fig. 4. Baboon 1 receiced 50 mg venom.

Fig. 7 demonstrates the results of the coagulation studies after denaturation of the venom at 56°C for one hour.

Platelet Aggregation Studies

One-tenth millilitre of venom was added to 1 ml normal human platelets, concentrated to 300 000/mm³ in each test.

Venom in a concentration of 100 μg/ml caused almost complete aggregation within 5 minutes; venom in a concentration of 50 μg/ml caused complete aggregation in 7 minutes, and venom in concentration of 30 μg/ml had no effect.

Studies on Baboons

Seven experiments were done.

Experiment 1 — baboons 1 and 2 received 50 mg venom: Both animals died within 45 minutes, with dramatic thrombocytopenia and severe fibrinolysis and very markedly elevated TRCHIIA (Fig. 4).

Experiment 2 — baboons 3 and 4 received 10 mg venom (Fig. 5): Severe fibrinolysis could be demonstrated by elevated TRCHIIA, prolonged thrombin time and increased fibrin plate lysis. The euglobulin lysis time was greatly prolonged as a result of the afibrinogenaemia with poor-quality clot formation. There was a dramatic, sustained fall in platelet count within 5 minutes, and the PTT became greatly prolonged. The haemoglobin and haematocrit levels fell rapidly, and one baboon died after about 2 hours, and the other after about 4 hours.

Experiment 3 — baboon 5 received 10 mg venom with 500 units of heparin stat, followed by 150 units every hour (Fig. 6): The same clinical picture as in the previous experiments was seen. Anuria developed after 40 minutes and the baboon died soon afterwards.

Autopsy reports on these 5 animals demonstrated the extreme susceptibility of the loose tissues of the gastrointestinal tract, with macroscopical and microscopical evidence of severe haemorrhage forming vascular lakes. There was no histological evidence of micro-clot formation and no microscopical aggregation of platelets in the blood vessels.

Experiment 4 — baboons 6, 7 and 8 received 100 μg, 200 μg and 500 μg of venom respectively (Fig. 7): Relatively few changes were noted, but there was a moderately prolonged PTT, and after the 500-μg dose hypofibrinogenaemia developed. The platelet count fell from 300 000/mm³ to 150 000/mm³ within 5 minutes. Fibrin plate lysis and TRCHIIA remained within normal limits.

Experiment 5 — baboon 9 received 1 mg of venom (Fig. 8): There was a moderate, immediate fall in blood pressure with a raised pulse rate. A fall in blood coagulation factors with a prolonged PTT, a reduced PA and PI and a fall in fibrinogen level were seen, but there was no evidence of fibrinolysis as tested by TRCHIIA, fibrin plate lysis and thrombin time. The platelet count fell within 5 minutes and remained low for at least 24 hours. This baboon developed moderately severe mucous membrane haemorrhages and haematuria.

Control animals — baboon 10 was anaesthetised and cut-downs were done on both femoral arteries. It was monitored in a similar fashion to the test animals and

received only a few millilitres of saline. The haematological studies were all satisfactory.

Baboon 11 (Fig. 9): This baboon received heparin in a dose sufficient to prolong the clotting time from 2½ minutes to 45 minutes, returning to normal within 6 hours.

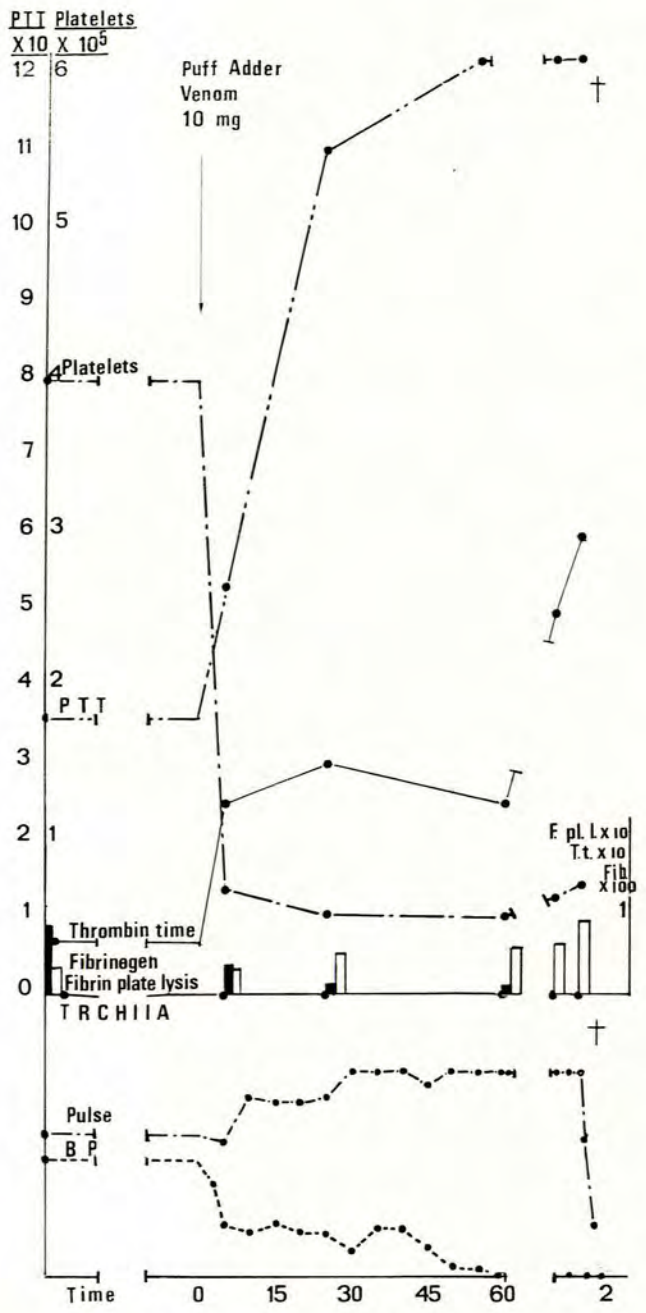

Fig. 5. Baboon 3 received 10 mg venom.

Experiment 6 — baboon 12 received 1 mg venom with 3 000 units of heparin as a single dose (Fig. 10): The baboon remained in a good clinical condition with no evidence of shock. The whole blood clotting time became normal after 6 hours, and 24 hours later routine studies were within normal limits. There was no evidence of mucous membrane haemorrhages or haematuria.

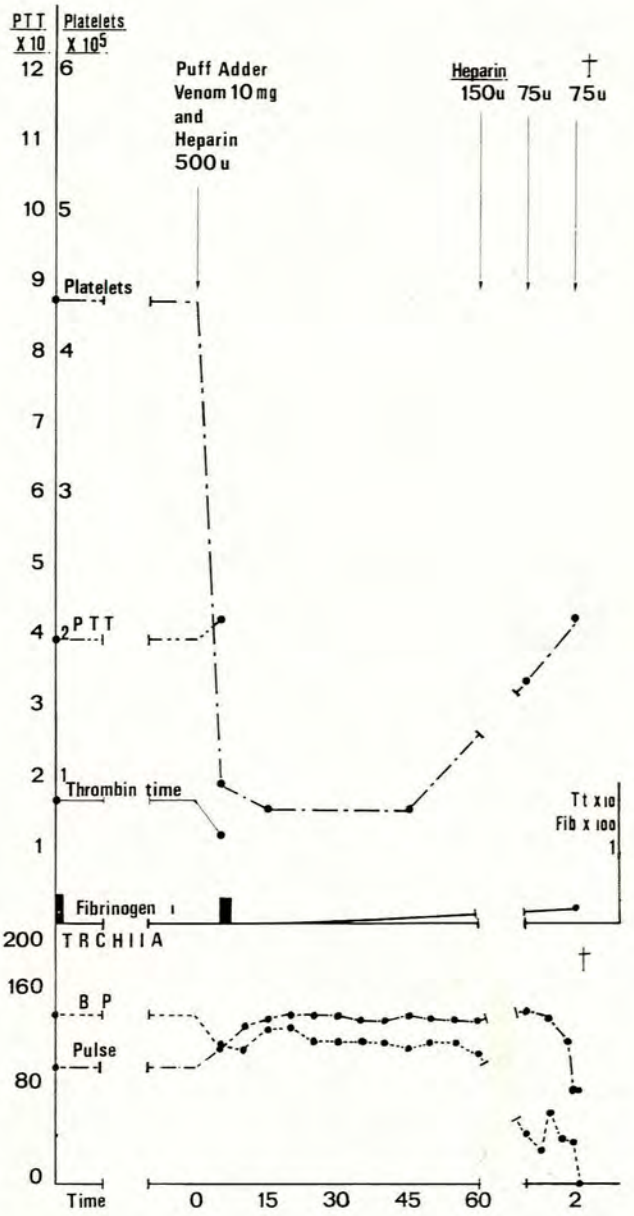

Fig. 6. Baboon 6 received 10 mg venom with heparin as indicated.

Experiment 7 — baboon 13 received 5 mg of venom and heparin 2 500 units 20 min after the venom (Fig. 11): There was a dramatic thrombocytopenia within 5 minutes and the fibrinogen level fell moderately. The thrombin time increased from 12 seconds to 25 seconds within 20 minutes, whereas the PTT, PA and PI showed no significant change within 20 minutes.

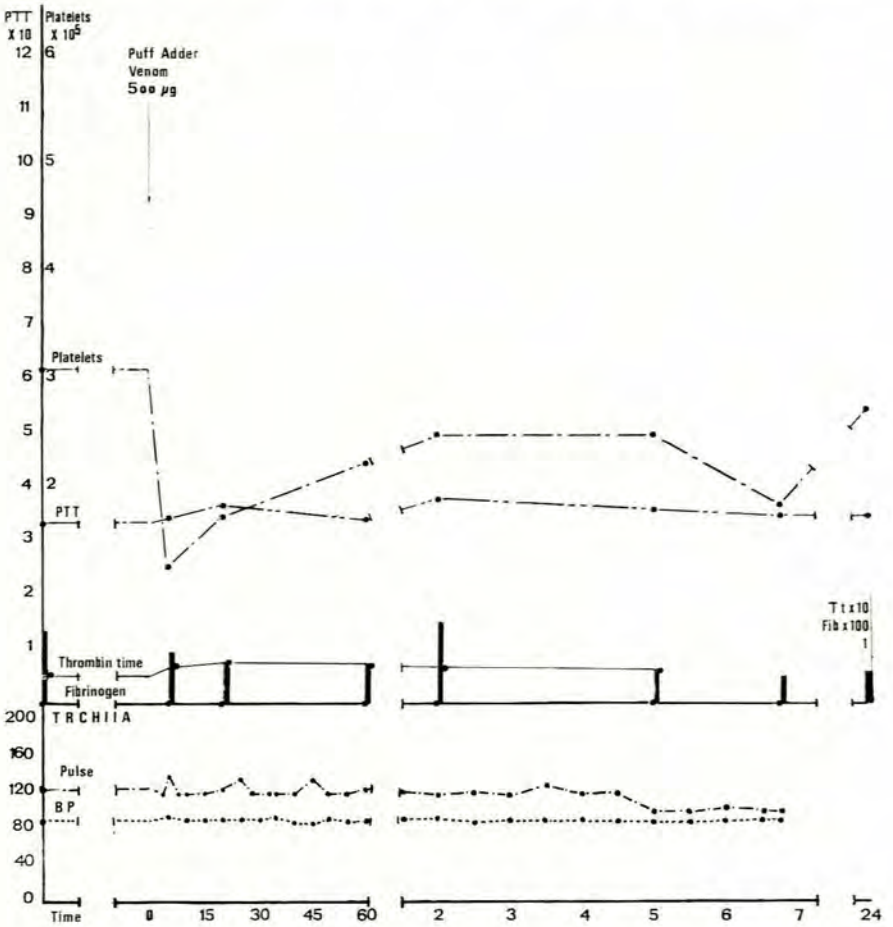

Fig. 7. Baboon 8 receved 500 µg venom.

Baboon 14 received 5 mg of venom and heparin 2 500 units immediately after the venom (Fig. 12): In both animals the blood became completely incoagulable after the heparin administration, and both went into a severely shocked state, developing bleeding gums and later extensive subcutaneous haemorrhages. They died within 4½ hours in a state of exsanguination. The dramatic thrombocytopenia was not prevented or corrected by heparin. They both developed haematuria and anuria. Schumm's test for methaem-albuminaemia was negative and there were no blood pigments in the urine.

With the fibrin plate lysis method increasingly severe fibrinolysis was demonstrated after the heparin administration. TRCHIIA tests were negative throughout the serum as well as the urine specimens.

DISCUSSION

The *in vitro* and *in vivo* haematological effects of the venom in the baboon with regard to its effect on coagulation, fibrinolysis and fibrinogenolysis were studied. It was also demonstrated that normal platelets are extremely sensitive to small doses of venom with irreversible aggregation occurring very rapidly.

Coagulation Studies in vitro

Fibrinogenolysis: On incubation of the venom with purified fibrinogen over 24 hours the thrombin clotting time was prolonged. This was associated with the digestion of fibrinogen as evidenced by the increased optical density at 280 Å, with incoagulability after 24 hours. The defective clotting was probably largely due to breakdown products of fibrinogen interfering with fibrin polymerisation. A large fraction of the enzymatic activity of the venom appeared to be heat-stable (56°C for 1 hour), but a small heat-labile fraction was also present. This should be further investigated.

Coagulation studies: The mode of action was primarily on the intrinsic blood coagulation mechanism, as evidenced by a prolonged PTT and clottable fibrinogen test with the PA and PI only moderately prolonged. It was again demonstrated that the greatest proportion of the venom is heat-stable, but there was a small heat-labile fraction as well.

Denaturation in relation to known enzymes: The lyophilised snake venom used in our experiments contained 77% soluble protein. Delpiérre[11,12] studied the chemical and enzymatic properties of three venoms. All three venoms tested (*Bitis arietans, Bitis gabonica* and

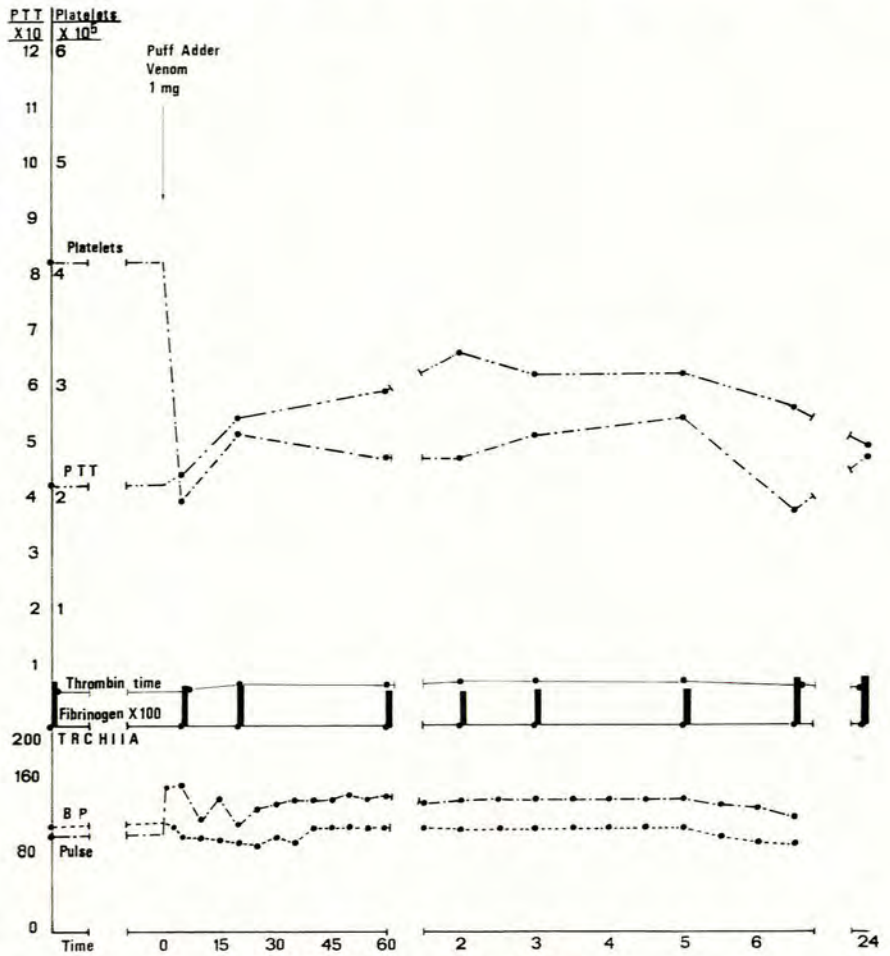

Fig. 8. Baboon 9 received 1 mg venom.

Causus rhembearus) were found to be active in catalysing the hydrolysis of casein and urea-denatured haemoglobin, with the puff-adder venom the most active, with an optimal pH of 10. Proteolytic and amino acid esterase enzyme activities can be separated by means of exchange and gel filtration chromatography. A considerable body of evidence exists to suggest that proteolytic activities are linked to the blood coagulation, the haemorrhagic effects and the severe necrotic lesions associated with the erectile-fanged snakes.[1,2]

The three major protease activites (A, B and C) from puff-adder venom were further separated by Van der Walt and Joubert[13] by gradient elution from CM-cellulose, and protease A was further purified by Sephadex G-75 gel filtration. It was found to have a molecular weight of 21 400 and it showed a tendency to undergo association, particularly at higher concentrations. The change in apparent molecular weight of protease A with decreasing concentration suggests a system of macromolecules which dissociate on dilution. Protease with a narrow specificity is of great value in the amino acid sequence determinations of proteins, but the protease A fraction of puff-adder venom was found to have a broad specificity, attacking mainly the amino end of hydrophobic amino acid residues.[14,15]

Other venoms: Tu *et al.*[16-18] undertook a systematic study of a wide variety of snake venoms, and reported on proteolytic, esterolytic, trypsin inhibitors, thrombin-like, trypsin-like, chymotrypsin-like and acetylcholinesterase-like enzymes.

Recently, with the hydrolysis of synthetic substrates, it has been shown that the proteolytic enzyme present in *Crotalidae* and *Viperidea* venoms is neither trypsin nor thrombin.[19-21]

Platelet Aggregation Studies

Normal human platelets (platelet-rich plasma) demonstated an extreme susceptibility to puff-adder venom. It seems as if puff-adder venom has a direct irreversible aggregating effect on human platelets, probably through ADP.

This aggregating effect of the venom was dose-related and was again demonstrated during the *in vivo* studies. All the animals given a dose above 1 mg demonstrated

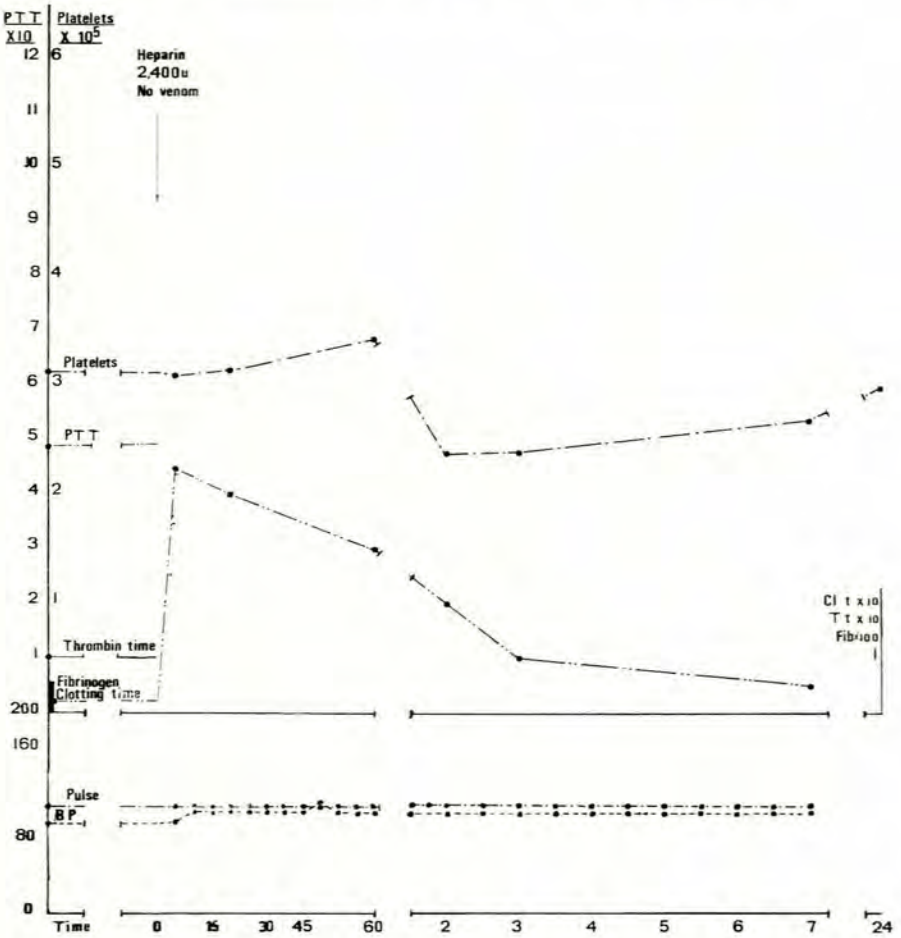

Fig. 9. Baboon 11 served as heparin control and received no venom.

a dramatic thrombocytopenia within 5 minutes, and this was sustained for at least 24 hours. This effect could not be prevented by the use of heparin *in vivo*.

In vivo Studies on Baboons

Massive dose: In normal adult baboons minute quantities of puff-adder venom caused severe shock and rapid death. All the animals given a dose of over 5 mg of venom showed a dramatic thrombocytopenia within 5 minutes and a fall in plasma fibrinogen. With a dose of 50 mg of venom fibrinolysis could be demonstrated and the blood became incoagulable. At autopsy there were extensive haemorrhages, particularly into the mucosa of the gastro-intestinal tract, but no evidence of microscopic thrombi formation. Failure to detect intravascular clots in envenomed humans and animals cannot be used as an argument against intravascular clotting, since it has been well documented that the clots may be dissolved ante- or postmortem by a fibrinolytic mechanism.[22,23] The rapid death of the animals was due to severe internal haemorrhage.

Smaller dose: The most prominent effect again appeared to be on the intrinsic coagulation mechanism, with hypofibrinogenaemia and transient thrombocytopenia. There was no evidence of fibrinolysis and it was unlikely that the changes could be ascribed to a generalised Schwartzmann reaction.

Effect of heparin: Heparin treatment considerably decreased the severity of the thrombocytopenia occurring in guinea pigs after moderate doses of *Echis coloratus* venom, but in no instance was it able to restore the platelet count to normal. Heparin, also in larger amounts, did not prevent rapid haemorrhagic death after administration of higher doses of venom, such as 200 - 400 μg. This is ascribed to the action of a vessel wall-damaging agent.[20] Heparin did not seem effective in *Agkistrodon rhodostoma* envenomation.[22]

Supporting evidence for the production of venom-induced incoagulability by intravascular clotting is obtained by experiments with heparin. It was shown to be effective both in preventing the afibrinogenaemia and in correcting it when administered after the venom injection in experimental envenomation with Russell's viper venom,[19] *Echis carinatus* venom[24] and with *Echis coloratus* venom.[19,20]

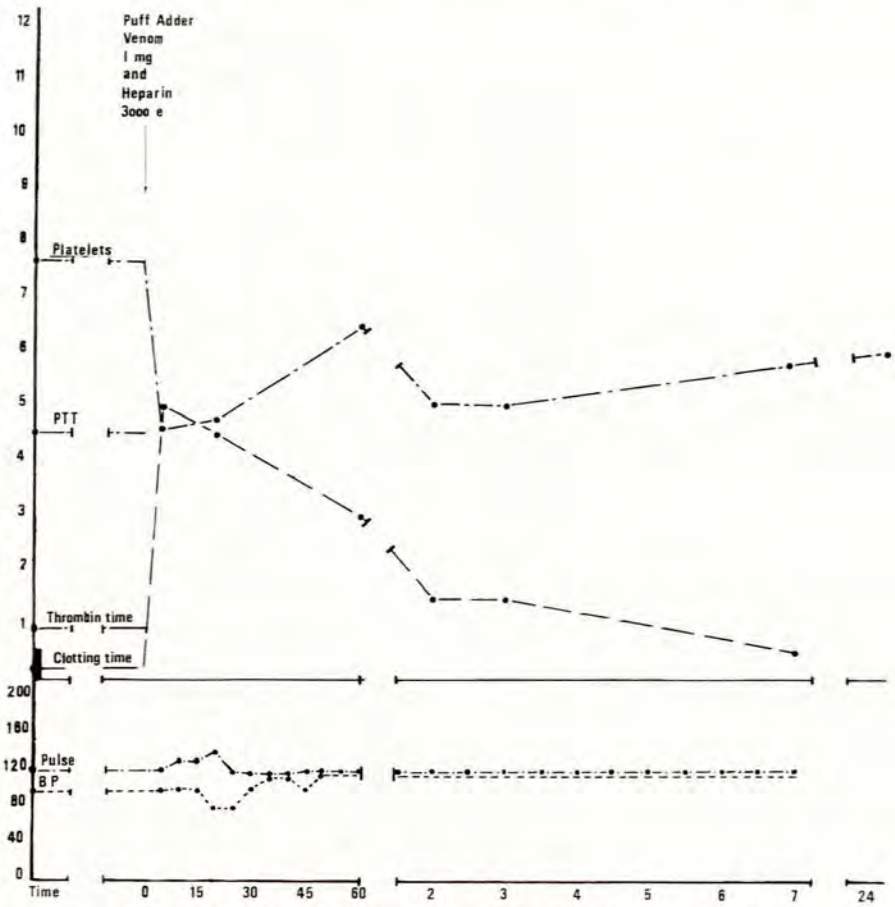

Fig. 10. Baboon 12 received 1 mg venom with heparin immediately.

In our studies the baboons in experiments 5 and 6 both received 1 mg of venom, but the second baboon also immediately received a large dose of heparin, and his clinical condition remained very satisfactory. There was no evidence of shock and the mucous membrane haemorrhages and haematuria so prominent in the baboon without heparin were completely absent. Both animals demonstrated thrombocytopenia within 5 minutes, and platelets remained low in the first baboon, whereas in the second animal they returned to normal after 24 hours.

The single massive dose of heparin given simultaneously with the venom appeared, on clinical evidence, to have a beneficial effect.

CONCLUSION

The effect of puff-adder venom in the baboon appears to be related to the dose. Venom in massive doses (5 mg or more) leads to dramatic thrombocytopenia, delay in the intrinsic clotting mechanism, fibrinolysis, fibrinogenolysis *in vitro* and multiple haemorrhages with rapidly developing shock. Heparin in large doses has no protective value and is contra-indicated.

With smaller doses of venom (1 mg or less) there is evidence of consumption of the blood coagulation factors, with platelet concentration still showing an initial moderate fall. Heparin may be beneficial in these cases, and might even have a direct effect on the platelets by preventing aggregation. This effect of heparin should be further investigated. It may have a place in treating mildly affected cases where absorption into the blood has been slow, when no antivenene is available, or the patients are sensitive to serum.

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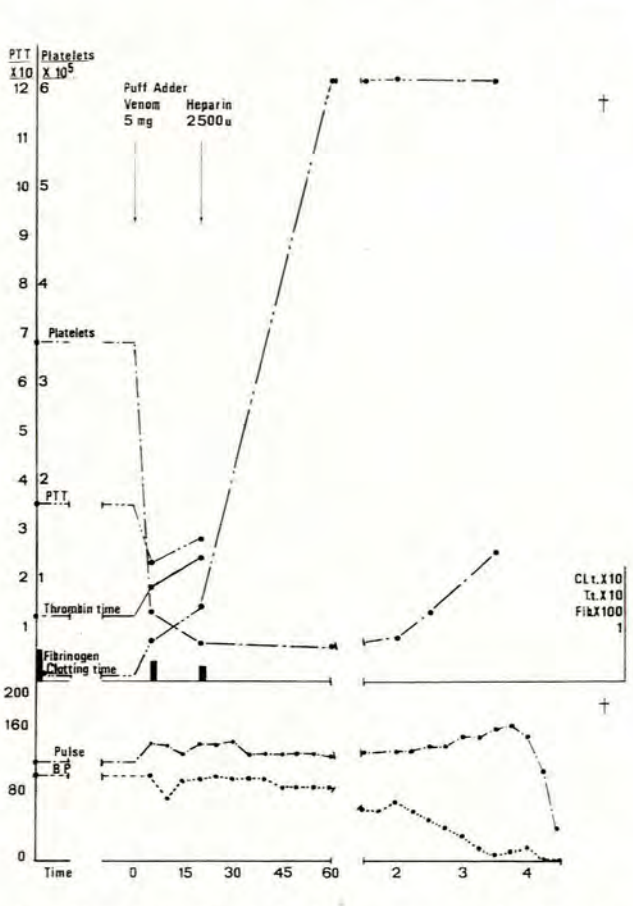

Fig. 11. Baboon 13 received 5 mg venom with heparin 20 minutes later.

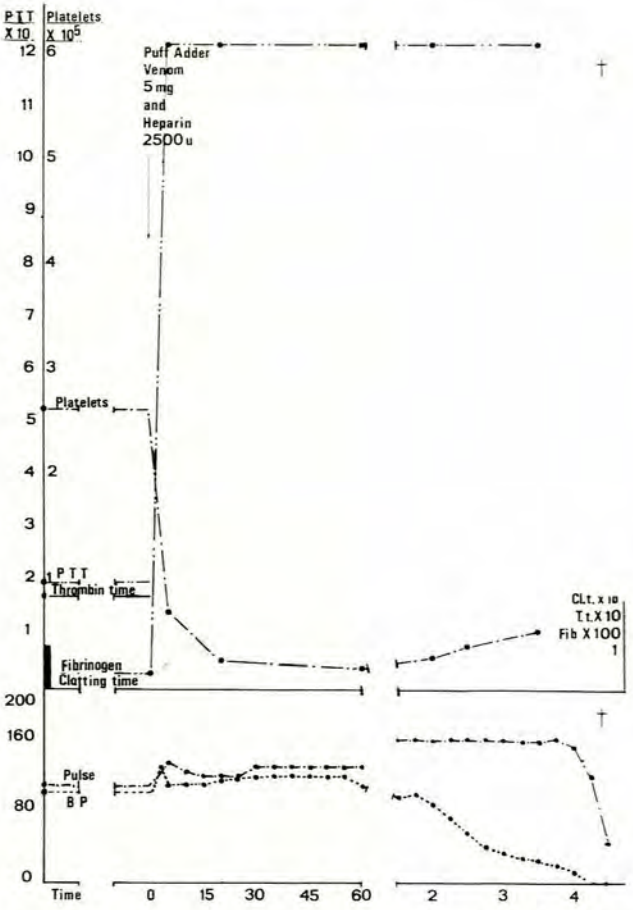

Fig. 12. Baboon 14 received 5 mg venom with heparin immediately.

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