Anticoagulant Action of Protamine Sulphate.* (23963)

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It has been known for a long time that protamine sulphate has an anticoagulant action (1,2). Ferguson(3) showed that protamine sulphate retarded or prevented conversion of prothrombin to thrombin in presence of brain but accelerated formation of fibrin from fibrinogen by thrombin: he called the latter action fibrinoplastic. In higher concentrations protamine precipitates fibrinogen(3,4) and it has been suggested that this is its principal anticoagulant effect(4): this reaction is reversible(5). Tocantins(6) showed that protamine sulphate prolonged the one-stage prothrombin time and believes that it is a true antithromboplastin as well as antiprothrombic. Portmann and Holden(7) however were unable to demonstrate an antithromboplastic action of protamine. The following study was undertaken to analyse the fundamental mechanisms involved in the inhibitory action of protamine sulphate with particular respect to blood thromboplastin generation.

Materials and methods. Thromboplastin generation test (TPG) was performed by the technic of Biggs and Douglas(8). *$Al(OH)_3$ adsorption* was performed by the method of Biggs and Macfarlane(9). *Serum factors* adsorbed by this technic were eluted with phosphate buffer (pH 8): this eluate is relatively free of inhibitors(10). *AHF* was prepared by the 33% saturation of $Al(OH)_3$ treated plasma(9), this material also contains fibrinogen and some factor V but is relatively free of antiaccelerator activity(11). *Partial thromboplastin times* (PTT) were performed by the method of Langdell, *et al.*(12), modified by the addition of protamine sulphate immediately before the addition of the final calcium. *One-stage prothrombin times* were determined by mixing 0.1 ml normal plasma, 0.1 ml fresh thromboplastin solution (Difco), 0.1 ml protamine sulphate solution and 0.1 ml 0.025M $CaCl_2$ in the order named. *Thrombin-fibrinogen times* were performed by adding 0.1 ml of bovine thrombin (10 units/ml) to a mixture of 0.2 ml of the 33% ammonium sulphate fraction of $Al(OH)_3$-treated plasma and 0.1 ml protamine sulphate or saline; the protamine sulphate was added 5 seconds before the final addition of thrombin. *Protamine sulphate* (Lilly) was used and diluted with saline buffered with imidazole (pH 7.2). Concentrations of protamine sulphate in all instances refer to concentration of protamine sulphate in the final clotting mixture except when otherwise stated.

Results. Effect of varying concentrations of protamine sulphate on one-stage prothrombin time, PTT and TPG. The PTT test is a slightly more sensitive index of the anticoagulant action of protamine sulphate than the one-stage prothrombin time and TPG test in respect of maximum yield (reflected by minimal substrate clotting time) (Table I). The protamine sulphate however was found to diminish the rate as well as the yield of blood thromboplastin formation although the action

TABLE I. Effect of Protamine Sulphate on Partial Thromboplastin Time (PTT), Thromboplastin Generation Test (TPG), One-Stage Prothrombin Time (1-Stage PT) and Thrombin-Fibrinogen Time.

Conc. protamine sulphate in incubation mixture, μg/ml	PTT	TPG, min substrate clotting times	1-stage PT	Thrombin-fibrinogen time
	← Sec. →			
125	200	32	41	Floc.*
62.5	140	21	32	5
25	125	16	25.5	6
15	108	15	21.5	7.2
7.5	102	13	19.0	8
3	98	9.8	17.8	8.1
2	93	8.3	16.2	8
1.5	90	8.0	15.2	8.2
1	82	8.1	15.4	8.2
.5	79	8.0	15.1	8
Saline control	80	8.2	15.2	8

* Flocculation.

* This investigation was supported by grant H-3536 from Nat. Heart Inst., N.I.H., Public Health Service.

Since submitting this paper very similar experimental findings were reported at Annual Meeting of Fed. Soc. for Exp. Biol. and Med., April, 1958 at Philadelphia, by Dr. J. N. Shanberge.

TABLE II. Effect of Protamine Sulphate on Rate of Thromboplastin Generation.

Conc. protamine sulphate in incubation mixture, μg/ml	Min substrate clotting time, sec.	Period of incubation required for min time, min.
Saline control	8.1	3
2	8.5	4
3	8.3	7
7.5	7.9	9
15	9.4	13

on rate was not always apparent due to normal deterioration of fully formed blood thromboplastin after several minutes. The effect on the rate was best brought out by substituting serum eluate and 33% ammonium sulphate fraction for normal serum and $Al(OH)_3$ treated plasma in the TPG test. This test system is relatively free of inhibitors and deterioration of fully formed thromboplastin is delayed. Using this system (Table II) it was found that concentrations of protamine sulphate in the incubation mixture ranging from 7.5 μg/ml to 2 μg/ml which was the lowest effective concentration, diminished rate but not yield of blood thromboplastin formation. Higher concentrations affected both yield and rate. In further experiments it was shown that the addition of extra amounts of $Al(OH)_3$-treated plasma, serum or platelets to the TPG test diminished the inhibitory effect of the protamine sulphate but these findings are difficult to evaluate since the control TPG mixture containing saline instead of protamine sulphate also showed increased coagulant activity. Ferguson† has found that $BaCO_3$ adsorbed beef serum diluted 1 in 10 has an antagonistic effect on the action of protamine in respect to the one-stage prothrombin time. This work was confirmed and it was also found that adsorbed beef serum would correct the defect in the TPG test produced by protamine sulphate.

Effect of preincubation of protamine sulphate with formed blood thromboplastin. A thromboplastin generation test was set up using 1 ml of each of the following reagents in the usual concentrations: $Al(OH)_3$-treated plasma, serum, platelets and calcium chloride. When the coagulant activity of the mixture reached a maximum, it was divided into 2 equal 1 ml parts. To one of these was added 0.2 ml of saline and to the other the 0.2 ml of protamine sulphate to give a final concentration of 7.5 μg/ml, this concentration of protamine had been previously shown to produce a significant diminution of yield of blood thromboplastin (minimum substrate clotting time of 15 seconds with a normal control of 10 seconds) when preincubated with the TPG test reagents used in this particular experiment. Immediately before and after the addition of protamine or saline and at subsequent 5 minute intervals samples were removed from each of the 2 mixtures and tested for coagulant activity. No significant differences between the two systems were obtained; the minimum substrate clotting times 1 minute after the addition of protamine sulphate or saline were both 10 seconds, showing that concentrations of protamine sulphate sufficient to produce marked inhibition on thromboplastin generation may have no effect on fully formed blood thromboplastin. The rate of deterioration was the same in both cases. If higher concentrations of protamine were added to the TPG mixture after the development of its maximal coagulant activity, the subsequent coagulant activity was less than that of the saline control but the difference was never appreciable. Thus, in one experiment a substrate clotting time of 13 seconds compared to 11 seconds in the saline control was obtained 1 minute after the addition of the protamine sulphate in a final concentration of 50 μg/ml or saline. Ten minutes later, both mixtures gave a substrate clotting time of 15 seconds, showing that the deterioration in the coagulant activity of the TPG mixture containing the protamine sulphate was not greater than that of the saline control.

Effect of adding varying concentrations of protamine sulphate to the substrate plasma in TPG. A thromboplastin generation test was set up as in the previous experiment using the same reagents but at no stage was protamine sulphate added to the incubation mixture. The coagulant activity of this mixture was tested at minute intervals and when the minimal substrate clotting was obtained the tubes containing substrate plasma were re-

† Personal communication.

TABLE III. Effect of Addition of Protamine Sulphate to Substrate Plasma in Thromboplastin Generation Test after Development of Maximal Activity.

Conc. of protamine sulphate in tube after addition of CaCl, μg/ml	One-stage "prothrombin" times (sec.) Blood thrombo-plastin	Diluted blood thrombo-plastin	Brain thrombo-plastin
125	15	20	36
62	13	17.2	25
25	11	17.2	19.5
15	11	15	17
7.5	11	15	16
3	11	15.5	15
Saline control	11	15	15

placed by tubes containing the same plasma substrate to which had been added saline or varying concentrations of protamine sulphate. The clotting times of these tubes were then determined in rapid sequence by the addition of blood thromboplastin and calcium as in the performance of one-stage prothrombin times. The experiment was concluded before the activity of the fully formed thromboplastin deteriorated. In a similar experiment the fully formed thromboplastin was diluted so as to have a coagulant activity equivalent to an extract of brain thromboplastin and the "one-stage prothrombin" times of mixtures of normal plasma and varying concentrations of protamine sulphate determined as before using the fully formed blood thromboplastin. The same experiment was then repeated using the brain thromboplastin instead of blood thromboplastin. The results (Table III) show that a concentration of protamine sulphate as high as 25 μg/ml has no effect on the "one-stage prothrombin" times performed using fully formed blood thromboplastin although this concentration will prolong the one-stage prothrombin time performed using a conventional tissue thromboplastin. The one-stage "prothrombin times" of both type of thromboplastin were prolonged by concentrations of protamine higher than 25 μg/ml.

Effect of preincubation of protamine sulphate with brain extract or plasma on one-stage prothrombin time. A concentration of protamine sulphate sufficient to lengthen the one-stage prothrombin time of normal plasma to 30 seconds was preincubated for varying periods up to 1 hour with brain. 0.2 ml aliquots of the mixture were removed at intervals and added together with 0.1 ml of .025M calcium chloride to 0.1 ml normal plasma and the clotting time recorded. A parallel experiment was performed using the same reagents but in which the protamine was incubated with plasma, aliquots removed at intervals and recalcified after the addition of brain so that the final clotting system was identical in the two experiments. It was found that the preincubation in both experiments produced no significant change in the clotting times which in all instances varied between 28 and 32 seconds.

Discussion. These findings show that protamine sulphate in relatively low concentrations diminishes both rate and yield of blood thromboplastin; these concentrations have no effect on fully formed blood thromboplastin and do not inhibit the reaction between *blood* thromboplastin and prothrombin in the substrate clotting tubes in the TPG test. In such low concentrations protamine sulphate is therefore antithromboplastinogenic and not antithromboplastic or antiprothrombic with reference to the action of blood thromboplastin. However low concentrations of protamine sulphate prolonged the one-stage prothrombin time performed by conventional methods as was first shown by Tocantins(6). At present, only factor V, Stuart factor and possibly factor X appear to be essential components of *both* the one-stage prothrombin and TPG test systems and as protamine sulphate is inhibitory in both these systems, it is possible that it acts specifically against one or more of these coagulation factors. The present status of factor X is now in doubt (13). Stuart factor affects yield rather than rate of blood thromboplastin formation while factor V affects both rate and yield so that on this hypothesis protamine sulphate would be more likely to act against factor V than Stuart factor.

The finding that the anticoagulant actions of protamine sulphate can be neutralized by $BaCO_3$ adsorbed beef serum which is rich in factor V but believed to be deficient in all other known coagulation factors appears to be further evidence supporting the hypothesis

that protamine sulphate is in fact an anti-factor V but the author agrees with Ferguson (personal communication) that this interpretation is unjustified since such serum is an extremely complex reagent. The unrelated finding that the addition of extra platelets, $Al(OH)_3$ treated plasma or serum to a normal thromboplastin generation system preincubated with protamine sulphate tend to neutralize the effect of the latter should also be viewed in the same light and interpreted with extreme caution.

It is clear that protamine sulphate has more than one anticoagulant action and several workers have confirmed that very high concentrations cause precipitation of fibrinogen and that relatively lower concentrations have a fibrinoplastic action(3,7). The lowest concentrations of protamine required for the fibrinoplastic action are roughly equivalent to the lowest concentration necessary to interfere with the reaction between blood thromboplastin, plasma and calcium and this concentration is considerably higher than the minimal concentration affecting the yield of blood thromboplastin generation.

The inhibitory action of protamine in high concentrations on the reaction between fully formed blood thromboplastin, plasma and calcium appears an immediate one and is non-progressive. It is possible that this action of protamine is both antiprothrombic and antithromboplastic as suggested by Tocantins(6) but other interpretations are possible.

Summary. Protamine sulphate in low concentrations affects both rate and yield of blood thromboplastin but has no effect on formed thromboplastin. Relatively higher concentrations inhibit a reaction between blood thromboplastin, prothrombin and calcium.

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Received March 14, 1958. P.S.E.B.M., 1958, v98.

Effect of Chlorothiazide on Vascular Reactivity.* (23964)

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Chlorothiazide (Diuril) has been recently introduced as an orally effective diuretic agent (1). Clinical studies(2,3) have indicated a definite anti-hypertensive effect of this compound, and the suggestion has been made that it may have a direct hypotensive action(4). Thus far, however, there has been no indication of a mechanism whereby chlorothiazide might act to reduce blood pressure directly. Therefore, the present study was undertaken to determine the effect of this substance upon vascular response to certain pressor agents.

* Aided by USPH grants.

Methods. 42 experiments were performed on adult mongrel dogs. They were anesthetized with sodium pentobarbital (30-35 mg/