Brit. J. Pharmacol. (1949), **4**, 135.

A STUDY OF THE TOXICITY OF THE PROTAMINE, SALMINE*

BY

L. B. JAQUES

From the Department of Physiology, University of Saskatchewan, Saskatoon, Canada

(Received October 26, 1948)

The protamine, salmine, has become widely known in the combined form, protamine-insulin. It has been generally assumed that the protamines are non-toxic. Vartiainen and Marble (1941) found that the median lethal dose of salmine subcutaneously in mice and rabbits was 200–300 mg./kg. and concluded that it was non-toxic in ordinary doses—a view supported by its widespread clinical use with no allergic or other side-actions. Reiner, de Beer, and Green (1942) found an LD50 of 94 mg./kg. in mice with protamine (presumably salmine?). Ahlstrom and von Euler (1946) have reported essentially similar results with rats and guinea-pigs, using salmine and also the related protamines, clupein and scombrin.

A second possible clinical use of protamine is as an antidote for heparin, first suggested by Chargaff and Olsen (1937) and reported by Jorpes, Edman, and Thaning (1939). As the protamine for this purpose will probably be given intravenously, it raises the question of possible toxicity by this route. Thompson (1900) reported that the intravenous injection of the protamine, clupein, gave markedly toxic reactions in the narcotized dog, 15–18 mg./kg. killing the animals. The injection of smaller amounts of protamine caused a definite and relatively prompt fall in blood pressure, which returned to normal in 25–30 minutes. In addition to the fall in blood pressure, the respirations became markedly increased in rate and depth, and were followed by a period of apnoea. Thompson also noted a delayed clotting of the blood and a leucopenia.

Jappelli (1933) confirmed Thompson's results with salmine using the unanaesthetized dog, as did Jaques, Charles, and Best (1938); 5 mg. salmine sulphate per kg. caused a pronounced fall in blood pressure and dyspnoea. However, they found that this protamine had relatively little effect on guinea-pigs, and this led to the present investigations. Shelley, Hodgkins, and Visscher (1942) reported that intravenous or intracardiac injection of 120 mg./kg. of protamine into rats and guinea-pigs caused death in three minutes with typical anaphylactoid symptoms. The same dose subcutaneously caused death in approximately three hours. As indicated below these results of Shelley *et al.* are not in serious disagreement with those obtained by Jaques, Charles, and Best and by Vartiainen and Marble.

* Read in part before the British Pharmacological Society, London, July 26, 1947.

Materials and Methods

The protamine used was a sample of salmine sulphate supplied by the Connaught Medical Research Laboratories, University of Toronto. It was judged to be free from histamine as it had no effect on the isolated guinea-pig ileum. While the major part of the study has been conducted with one large sample of protamine, three other batches were used in the earlier experiments with the same results. The protamine was dissolved in saline in a concentration of 10 mg. per ml. For large doses 20 mg. per ml. solutions were used; these were somewhat milky, but, by drawing the solution up into the syringe and forcing it out a few times, a uniform suspension of the protein could be obtained. The protamine solution was injected into either the jugular or the femoral vein, and unless otherwise indicated the injection time was 11–14 seconds. Unless otherwise indicated, all animals were maintained under pentobarbitone anaesthesia.

Cell counts were made in an improved Neubauer haemocytometer. Platelets were counted using the citrate diluting fluid described by Fidlar and Waters (1941). Values for both red cells and platelets are given for the same fields counted. Correction of the platelet count for dilution errors by use of the red cell count was calculated, but, as this did not result in any significant change in values, it has been omitted in the values reported. Blood histamine was measured on the guinea-pig ileum after isolation by the method of Code (1937) and also by direct assay of plasma, the technique of Anrep, Barsoum, and Ibrahim (1947) being used. It has been established that this technique can be satisfactorily applied to plasma (Scroggie and Jaques, 1948). Identity tests for histamine were conducted on the blood pressure of the cat after administration of atropine. In certain experiments, glassware was treated with a silicone, General Electric Dri-Film No. 9987, as described by Jaques, Fidlar, Feldsted, and Macdonald (1946). The glycogen used was obtained from Eastman Kodak, Ltd.

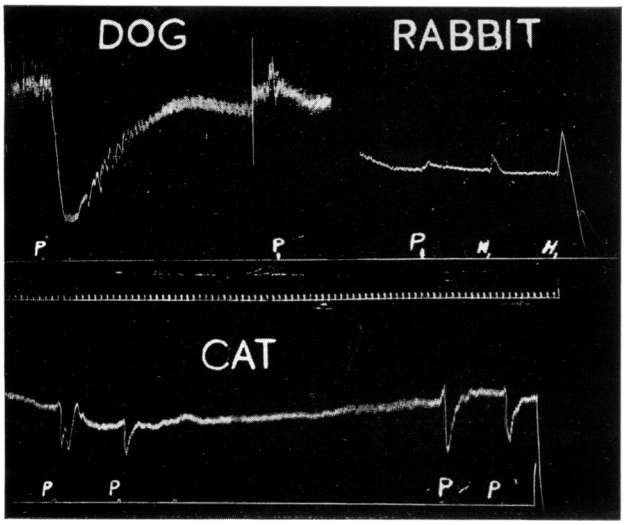

FIG. 1.—Effect of protamine on blood pressure. P = 5 mg. protamine/kg. intravenously. Time marker = 30 seconds. Dog: Initial B.P.—136 mm. Hg. B.P. started to fall after 45 seconds, minimum (30 mm. Hg) reached in 2 minutes 10 seconds; 3¼ hours elapsed between first and second injection. Rabbit: Initial B.P.—80 mm. Hg. H = 0.2 and 5 mg. of histamine. Cat: Initial B.P.—80 mm. Hg. Protamine at 3.05, 3.10, 3.36, and 3.41 p.m.

RESULTS

The effect of intravenous injection of protamine

In the previous study (Jaques, Charles, and Best, 1938) it was reported that the intravenous injection of protamine into normal dogs caused gastro-intestinal symptoms—retching, vomiting, diarrhoea, and cramps. In the anaesthetized dog, 5 mg. per kg. of protamine caused a marked fall in blood pressure. Associated with this was a marked hyperpnoea. These effects were also obtained with preparations from thymus (thymus histone) and from blood (globin) which, like protamine, precipitate insulin and neutralize heparin. In the present study, 5 mg./kg. of protamine was injected into animals under pento-barbitone anaesthesia and the blood pressure recorded (Fig. 1). Injection of protamine in the dog caused a very marked fall in blood pressure. A second injection three hours and fifteen minutes later had little effect on the blood pressure.

20 mg. of protamine were injected intravenously into a rabbit (2.8 kg.) under urethane anaesthesia (Fig. 1). There was a slight rise in pressure; 0.20 mg. of histamine caused a somewhat higher rise. This slight rise in pressure was not consistently obtained with protamine.

In contrast to the effects in the rabbit and dog, 5 mg./kg. of protamine injected intravenously in the cat caused a short sharp fall in blood pressure of 36 mm. Hg; this result was obtained each time the protamine was injected and hence did not resemble the reaction in the dog discussed in detail below.

Jaques, Charles, and Best (1938) previously reported that intravenous injection of protamine into guinea-pigs had no significant effect in doses up to 50 mg./kg. This is remarkable in view of the toxicity of protamine in the dog. In the present work guinea-pigs were given 50 mg./kg. of protamine intracardially. These animals showed mild anaphylactoid symptoms (snuffling, weakness of hind legs and then forelegs), but all recovered in an hour, with no deaths. Similar reactions were obtained in a series of guinea-pigs given 100 mg. of dialysed Witte peptone intravenously. It appears likely that this is the earlier stage of the effect observed by Shelley, Hodgkins, and Visscher (1942), who established that 120 mg./kg. would be lethal in three minutes in the guinea-pig. As discussed later, this toxicity for the guinea-pig at dosage levels ten times that in the dog may be due to a quite different mechanism.

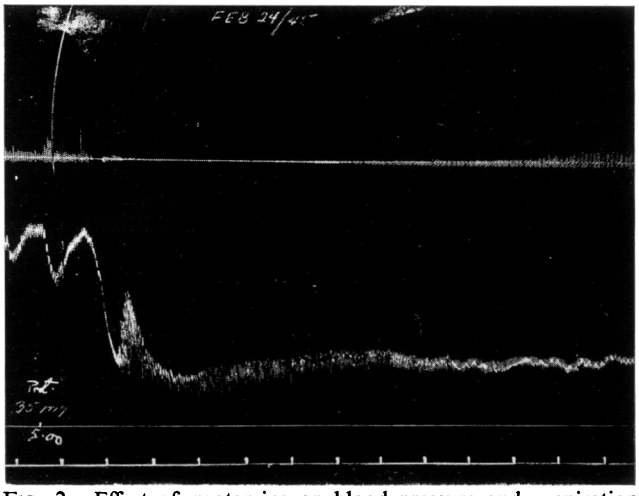

FIG. 2.—Effect of protamine on blood pressure and respiration in the dog. Protamine—5 mg./kg. Time marker—15-second intervals. Initial B.P.=134 mm. Hg.

The toxicity of protamine intravenously in the dog

Since protamine is most toxic for the dog, further studies were conducted on this animal. The immediate effect of 5 mg./kg. of salmine intravenously on blood pressure and respiration of the dog is shown in more detail in Fig. 2. There is a slight hyperpnoea followed by a period of apnoea, which is then succeeded by normal respiration. The protamine preparations did not give the marked dyspnoea previously reported by Thompson (1900) with thymus histone and clupein. The apnoea appears with the initial hypotension. After a lag of thirty seconds, the blood pressure quickly falls to a minimum (30 mm. Hg), then recovers, reaching a level about 10 mm. Hg below the initial blood pressure in six to ten minutes. The initial blood pressure is not completely attained for an hour or more, but the period of hypotension has been measured only to the point where the blood pressure tracing became level again.

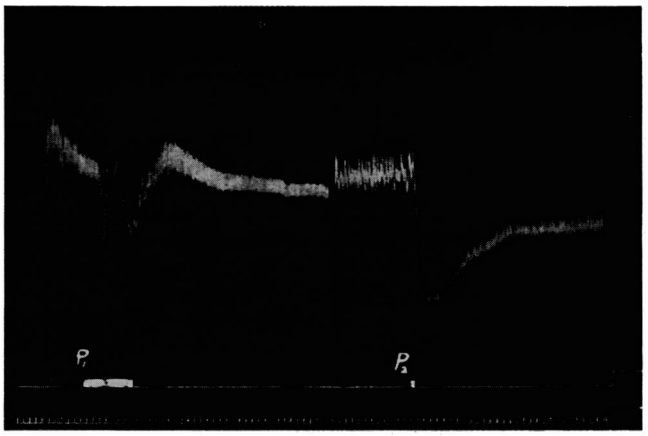

FIG. 3.—Effect of protamine on blood pressure. 5 mg./kg. protamine intravenously. P_1 — injected in 4½ minutes; P_2—injected in 15 seconds, 70 minutes after P . Time marker —15-second intervals. Initial B.P. = 184 mm. Hg.

The effect of various dose levels of salmine administered intravenously in the dog is reported in Table I: 0.1 and 0.5 mg./kg. had no effect on blood pressure although there was a slight effect on respiration; 1.0 mg./kg. appeared to be the minimal effective dose and it caused a transient fall in blood pressure. Doses of 5 and 10 mg./kg. gave the typical fall in blood pressure of short duration already described. With doses of 15, 20, and 35 mg./kg. the hypotension was of longer duration, but this was due to the slowness of complete recovery; marked hypotension persisted for a much shorter time (eight minutes with a 35-mg. dose), and the blood pressure reached half its initial value in 15 minutes (15 mg.), 5 minutes (20 mg.), and 70 minutes (35 mg.). Even with the highest dose (35 mg.) the animal recovered.

TABLE I

EFFECT OF PROTAMINE INTRAVENOUSLY IN THE DOG

Dog	Dose mg./kg.	Original blood pressure	Maximal fall in B.P.	Duration of hypotension	Change in respiration
C1	0.1	120	None	—	Shallow and rapid
C10	0.5	98	,,	—	—
C29	1.0	130	24	1′	—
C13	5.0	100	80	6½′	Cheyne-Stokes
C22	5.0	120	60	6′	Dyspnoea
C29	10.0	144	140	8′	Apnoea
C28	15.0	86	86	40′	Apnoea
C1	20.0	130	104	>40′	
C1	35.0	120	100	>1 hr.*	

* Normal blood pressure 6 days later

As illustrated in Fig. 1, a second injection of protamine in the dog is ineffective. This failure of response to a second dose lasts approximately four to six hours. In one animal a second injection after an interval of four hours produced a fall in blood pressure one-half that obtained with the first injection, and we have consistently obtained the original fall in blood pressure with a second injection twenty-four hours after the first.

As shown in Fig. 3, the rate of injection of protamine is a factor in developing shock in the dog: when 5 mg./kg. was injected over a period of 4½ minutes there was some irregularity in the blood pressure tracing but no typical fall. Seventy minutes later, the same dose of protamine was injected over a period of fifteen seconds, as in the other experiments, and the typical fall in blood pressure was obtained. Evidently the first slow injection of protamine was ineffective in causing the full fall in blood pressure and also failed to protect the animal against the effect of a second injection. It was of interest that the first injection had a marked effect on respiration. Marked dyspnoea was observed when one-third of the total amount of protamine had been injected. The fluctuations in blood pressure corresponded with the period of respiratory distress; this suggests that the respiratory effects of protamine can be dissociated from its depressor action.

The effect of pre-treatment of the dog on protamine shock

In order to investigate the nature of this action of protamine various agents were tested for their action on protamine shock (Table II). Mendel and Rudney (1944) have shown that protamine will inhibit the true cholinesterase at concentrations of an order which are physiologically significant. In two animals, the sensitivity to protamine was determined by the injection of 5 mg./kg. Twenty-four hours later, 0.02 mg./kg. of atropine was injected intravenously and five minutes later the injection of protamine was repeated. The same fall in blood pressure was obtained.

TABLE II

EFFECT OF PRE TREATMENT ON THE HYPOTENSIVE ACTION OF PROTAMINE IN THE DOG

Pentobarbitone anaesthesia; 5 mg. protamine per kg.

Dog	Pre-treatment	B.P. mm. Hg Before protamine	B.P. mm. Hg After protamine	Recovery time in min.
S-11	—	112	20	8
,,	Atropine, 0.1 mg.	130	46	4.5
S-12	—	100	40	3.5
,,	Atropine, 0.2 mg.	96	14	4.5
C-10	Heparin 5 mg./kg. 20′ before	144	36	7
C-1		110	25	>40
C-30		154	69	>40
C-19	Heparin mixed with protamine *in vitro*	158	55	18
C-29		171	157	—
C-29	25 mg./kg. protamine 70′ later	142	36	>35
M9-5	Dicumarol 2 days previously	130	30	13
M9-9		170	56	15
M9-11		130	13	18
M-81	Eviscerated dog	116	52	8.5
M-06	Cord section ..	77	23 (fall occupied 2′)	60
M-08	,, ,, ..	124	46 (fall occupied 1′)	1
M-10	,, ,, ..	76	27 (fall occupied 2½′)	2
M-82	Benadryl, 100 mg. i.v.(*) (approx. 12 mg./kg.)	168	26	12
M-83	,, ,,	166	32	12
M-97	,, ,,	107	67 (fall occupied 1¼′)	1
M-98	,, ,, (*)	132	117 (fall occupied 2½′)	0.5
M-99	,, ,, (*)	175	97	25

* 10 mg. protamine per kg. in these experiments

Since the clinical use of protamine intravenously is likely to be chiefly for the purpose of neutralizing heparin, the effect of heparin on its depressor action was studied. Three dogs (C-10, C-1, C-30) were given 5 mg./kg. of heparin intravenously twenty minutes before the protamine; this assured an excess of heparin over the protamine in the circulation. As shown in Table II, heparin did not modify the effect of protamine on blood pressure; indeed, the shock appeared to be slightly more severe. In two other animals the protamine was mixed with the heparin in the syringe and the precipitate of heparin-protamine compound was injected; the toxic action of protamine still appeared. However, with dog C-29, when the compound had stood in the syringe for a longer time and the precipitate had formed larger masses, there was no fall in blood pressure; this was evidently a true neutralization of the toxic action of the protamine since the later injection of protamine itself caused the typical fall in blood pressure.

As shown below, intravenous injection of protamine causes the destruction of platelets. The liberation of thromboplastin from the platelets might cause shock similar to that reported by Mylon, Winternitz, Katzenstein, and de Suto-Nagy (1942). However, in three dogs given dicumarol two days previously, there was a typical fall in blood pressure after protamine. Removal of the abdominal viscera, including the liver, by the method of Markowitz (1937), did not alter the effect of protamine on blood pressure (M-81; Table II).

The effect of cord section on the action of protamine was also studied. In a series of dogs the spinal cord was severed between C2 and C3. Artificial respiration was started and the protamine injected in the usual way. A slow fall in blood pressure occurred after cord section. Injection of protamine resulted in a further superimposed fall in blood pressure, with partial recovery. The fall in blood pressure did not resemble that after the injection of protamine in the normal animal. Instead the development of the depressor action was much retarded and the fall in blood pressure was very slow. In animals similarly prepared but in which complete transection was not accomplished, protamine gave the typical fall in blood pressure. The results suggest that cord section affects the toxic action of protamine, particularly the rapid fall in blood pressure.

The effect of benadryl on the response to protamine was also studied (Table II). Irregular results were obtained which resembled those of Wells, Morris, Bull, and Dragstedt (1945) on the effect of benadryl in anaphylactic and histamine shock.

They found that, while complete inhibition did not result, benadryl sometimes largely neutralized the effect of the antigen. De Cuyper (1946) has reported that antergan counteracted the action of clupein on blood pressure, and it may reasonably be concluded that protamine shock is modified by antihistamine drugs.

Blood changes in protamine shock

The blood concentration of a number of factors was determined in protamine shock in the dog. Protamine concentration in the blood was determined by titrating its anticoagulant activity with heparin. At the neutralization point of these two anticoagulants, the clotting time is normal (Table III). Under the conditions used, 25 μg. of protamine

TABLE III

TITRATION OF PROTAMINE WITH HEPARIN

0.5 ml. of blood added to 25 μg. of protamine plus varying concentrations of heparin in 0.5 ml. of saline. ∞ means no coagulation

Protamine (μg.)	Heparin (μg.)	Clotting time (min.)
25	3.0	16
	4.0	13
	4.5	7
	5.0	8
	5.5	8
	6.0	22
100	10.0	∞
	15.0	∞
	20.0	26
	25.0	∞
	30.0	∞
0	0	6.5

was neutralized by 4.5 μg. of heparin. An interesting observation was made in connexion with these tests; the sensitivity of the test is greatly increased by diluting the blood with an equal volume of saline as described by Jaques, Charles, and Best. Control experiments showed that this dilution not only increased the normal clotting time and the clotting time with added heparin but also increased the clotting time with added protamine tenfold. Estimations of salmine present in the blood after intravenous injection of the protamine are reported in Table IV; 95 per cent of the injected protamine had disappeared within five minutes of the injection. The slow disappearance of the remainder is presumably due to the hydrolysis of protamine by plasma enzymes (Hagedorn, 1938).

The histamine and heparin contents of the blood were determined before and after the injection of

TABLE IV

CONCENTRATION OF PROTAMINE IN THE BLOOD AFTER INTRAVENOUS INJECTION

Injection—5 mg./kg. = 50 μg./ml. of blood

Time after injection of protamine	Clotting time min.	Heparin required to reduce the clotting time μg.	Protamine equivalent μg./ml.
Dog 8			
0	15.0	0	0
6 min.	15.0	0.3	3.0
3 hr.	8.0	0.1	1.0
21 hr.	6.5	0.04	0.4
Dog 10			
0	10	0	0
5 min.	10	0.3	3.0
32 min.	17	0.1	1.0
2 hr.	8	0.05	0.5
4 hr.	7	0.03	0.3

protamine (Table V). No increase in blood histamine was detected after the injection of protamine. The value for the normal blood histamine in Table V was abnormally high, but no increase was detected in the blood of the other animals of the series which showed normal values. Although these results were negative they do not allow the conclusion that the

TABLE V

BLOOD CHANGES IN PROTAMINE SHOCK

Dog M94. Protamine: 10 mg./kg.

Time from injection	B.P. mm. Hg	Blood histamine μg./ml.	R.B.C. × 10^3/cu.mm.	Platelets × 10^3/cu.mm.
—	160	2.0	5,150	95
—	160	2.0*	—	—
20″	140	—	6,850	35
90″	40	1.8	7,000	30
135″	18	1.8	—	—
190″	21	1.8	—	—
15′	69	1.7	6,500	15

* Blood sample + protamine, 0.1 mg./kg.

observed effect of protamine on blood pressure was not due to the liberation of histamine; 30 μg./kg. of histamine gave a fall in blood pressure in the anaesthetized dog equivalent to 10 mg./kg. of protamine so that the amounts of histamine liberated into the blood to give the fall in blood pressure observed may be too small to be determined by the methods used. In tests for histamine in the cat after atropine, an indication of an unstable depressor substance (not histamine) in the dog's plasma was

seen in one animal at the moment of lowest blood pressure.

Heparin was estimated by the method of Jaques, Monkhouse, and Stewart (1949). Heparin in blood can be determined by this method in concentrations above 0.8 μg./ml. (0.1 international units). No heparin was found in the blood after the injection of protamine. In view of the affinity of protamine for heparin, it would be of interest to study the mast cells in order to discover whether protamine had become localized there, but this was not done in the present study. The injection of protamine had little effect on the red cell count. The increase in count shown in Table V was not a constant accompaniment of the action of protamine.

The thrombocytopenic action of protamine

The most marked effect of protamine on the blood constituents was the marked fall in the white cell and platelet counts (Tables V and VI).

TABLE VI

EFFECT OF PROTAMINE ON CELL COUNTS

Protamine—5 mg./kg. in each experiment

Minutes after injection	B.P. mm. Hg	R.B.C. $\times 10^6$	W.B.C.	Platelets $\times 10^3$	Platelets agglutinated per cent
DOG—7.3 kg.					
0	160	6.526	7,600	408	0
1½	46	7.504	1,600	30	39
4	140	6.894	2,600	27	39
17	140	7.029	7,900	113	26
RABBIT—2.8 kg.					
0		5.662	3,100	349	11
2⅓		5.681	3,700	367	9
8		5.506	3,400	371	1

The fall in the platelet count was observed twenty seconds after the injection, before there was any fall in blood pressure. The maximum thrombocytopenia, with a count about 10 per cent of the normal, was generally reached in two to four minutes at the time of the maximum reduction in blood pressure. The recovery of the platelet count was quite rapid, but lagged behind the return to normal of the blood pressure. There was usually a significant rise in the count in fifteen minutes and the count was largely restored within an hour.

As shown in Tables VI and VII there appears to be some connexion between the extreme thrombocytopenia and the fall in blood pressure produced by protamine in the dog. In the rabbit with no fall in blood pressure, there was no change in the platelet count. In several rabbits a fall did occur, but it did not amount to more than 10 per cent. With repeated injections of protamine in the dog, it will be evident from Table VII that after the first injection, accompanied by a fall in blood pressure, there

TABLE VII

EFFECT OF REPEATED INJECTIONS OF PROTAMINE ON BLOOD PRESSURE AND CELL COUNTS IN THE DOG

Time	B.P. mm. Hg	R.B.C. × 10^3/cu.mm.	W.B.C. per cu.mm.	Platelets × 10^3/cu.mm.
10.20 a.m.	—	6,843	10,000	290
11.45	145	5,879	7,300	319
11.50	Protamine—5 mg./kg.			
11.53.30	30	—	—	—
11.56	100	5,188	3,700	47
12.30 p.m.	140	6,606	3,600	87
2.00	140	6,438	4,600	196
3.10	140	6,363	18,400	222
3.13.30	Protamine—5 mg./kg.			
3.14.50	150	7,446	5,100	172
3.19.10	144	7,220	14,900	173
3.59	144	7,213	17,100	223
9.27	144	6,620	27,000	261
9.36.10	Protamine—5 mg./kg.			
9.38.40	150	6,944	9,100	160

was a pronounced decrease in the platelet count to 15 per cent of the pre-injection value. However, on the second and third injection, the count was decreased by only 22 and 39 per cent. In our studies on platelets we have found in general that a fall in the platelet count of 20 or 30 per cent is caused by many substances and is a general type of reaction, whereas a fall in the platelet count of 80–90 per cent is a more specific effect. Hence the thrombocytopenia after the second injection may be not only quantitatively but also qualitatively different from the first.

In addition to the thrombocytopenia, the intravenous injection of protamine also caused a leucopenia in the dog (Tables VI and VII), but, as with the platelets, the white cells were not affected in the rabbit. In contrast to the changes observed in the platelet count, with repeated injections of protamine in the dog there was a greater decrease in the leucocyte count on the second and third injections.

In order to determine whether it affected the platelets and white cells directly, protamine was mixed with blood outside the body and counts performed on the mixture. Since the properties of the platelets are markedly affected by anticoagulants, the silicone technique described by Jaques, Fidlar, Feldsted, and Macdonald (1946) was used. By this technique it is possible to keep blood for over

30 minutes without a decrease in the platelet count. The needles and syringes for drawing the blood were coated with this material. 5 ml. of blood were drawn and 1 ml. added to 0.1 ml. of protamine solution (50 μg.) in a silicone-coated beaker; 1 ml. of blood was also added to 0.1 ml. of saline in a similar beaker to act as a control. As shown in Table VIII, there was relatively little change in the platelet count in the control beaker in eleven minutes. The decrease in count observed was probably due to sedimentation causing difficulties in

TABLE VIII

EFFECT OF PROTAMINE ON BLOOD IN SILICONE

Clotting time of blood in silicone: >2 hr.

1 ml. of sample blood + time	R.B.C. × 10^6/cu.mm.	W.B.C. cu.mm.	Platelets cu.mm.
0.1 ml. saline 1′	5.794	14,500	288,000
0.1 ml. saline 11′	7.638	18,000	264,000
0.1 ml. protamine (50 μg.) 2′	7.075	15,900	314,000
0.1 ml. protamine (50 μg.) 12′	7.075	18,000	40,000

obtaining a representative sample, since the red cell and leucocyte counts showed a corresponding increase. However, in the sample with added protamine, there was a very marked decrease in the platelet count of 87 per cent, although there was no decrease in the white cell count. It is evident that protamine affected the platelets directly whereas it had no direct effect on the leucocytes. The decrease in the platelet count may have been due either to lysis of the platelets or to agglutination of the platelets with settling out of the platelet masses. This is possible both *in vivo* and in the silicone beaker, although the very marked decrease in the platelet count in the latter with no evidence of agglutinated platelets suggests that lysis has occurred. Actually the two phenomena are not necessarily distinct, since platelet agglutination is frequently followed by " viscous metamorphosis " and eventual solution of the platelets. Hence, while agglutination could be the chief factor *in vivo* and is illustrated in Table VI, this is probably succeeded by lysis in the silicone beaker.

The fact that the white cell count is not affected by protamine in silicone suggests that the fall in the white cell count which occurs *in vivo* is not a direct action of the protamine. Such a decrease in the white cell count occurs, however, whenever there is agglutination of platelets, as the white cells stick to the platelet clumps which are blocked in the capillaries. This suggests that agglutination of the platelets is the chief phenomenon on injecting protamine *in vivo*, and that the leucopenia is secondary to this.

Since an effect of protamine on the platelets could be detected *ex vivo* by means of the silicone technique, a direct comparison was made of the action of protamine on platelets *in vivo* and *ex vivo*. A sample of blood was taken and added to protamine and saline in silicone as before. Samples for platelet counts were taken after 10 sec., 2, 5, and 10 min. A corresponding quantity of protamine (5 mg./kg.) was injected intravenously and samples for platelet counts taken at the same time intervals. The experiment was repeated three hours and ten hours later. The results are shown in Table IX. It will

TABLE IX

COMPARISON OF THE EFFECTS OF PROTAMINE ON PLATELETS *in vivo* AND *ex vivo*

Protamine = 5 mg./kg. = 0.05 mg./ml. of blood

Time	Platelets × 10^3/cu.mm.		
	Ex vivo	Saline control	*In vivo*
0	—	—	120
10″	90	110	80
2′	70	115	25
5	50	110	20
10′	—	—	20
120′ (0)			90
10″	65	105	65
2′	60	100	30
5′	45	90	30
10′	50	—	65
10″	70	65	—
2′	40	70	65
5′	40	60	60
10′	45	—	25
60′			20

be evident that the thrombocytopenia observed after the injection of protamine also occurred at the same time *ex vivo*, although, since the fall was not as great, part of the thrombocytopenia after the injection of protamine is due to filtering out of platelet clumps in the capillaries. A difficulty in interpretation in this experiment, compared to that of Table VII, was caused by the fact that the initial platelet count of this animal was low and the recovery of the count much poorer, so that, while the decrease in the count was less after the second and third injection, the thrombocytopenia was just as severe.

The effect of glycogen on the toxicity of protamine

From the above it is evident that the agglutination of platelets and accompanying white cells is inde-

pendent of the effect of protamine on blood pressure, at least as far as the moderate thrombocytopenia occurring on a second and third injection of protamine. However, it is possible that the extreme fall in the platelet count after the first injection is related to the fall in blood pressure at this time. As mentioned previously, the fall in the platelet count actually begins before the fall in blood pressure. However, this does not give us any indication whether the fall in blood pressure is the cause or the result of the platelet agglutination associated with the marked fall in the platelet count. Rocha e Silva, Grana, and Porto (1945) found that the intravenous injection of glycogen will produce a temporary disappearance of the platelets from the blood, and they have used this as a method of studying the contribution of the platelets to various types of anaphylactoid shock. Four dogs were therefore given glycogen (1 g./kg.) intravenously before the injection of protamine (Table X). A complete

TABLE X

EFFECT OF THE INJECTION OF GLYCOGEN ON THE ACTION OF PROTAMINE

Expt.	Platelet count $\times 10^3$/cu.mm.			B.P., mm./Hg		
	Before glycogen	Before protamine	After protamine	Before protamine	After protamine	Recovery time
M–64	402	119	114	107	28	6′
M–00	75	·9.2	4.3	98	82	20″
M–02	110	68	3.8	102	28	12′
M–11	108	9	5	159	143	15″
M–11*	—	80	8	116	61	20″

* 2 hours after first injection

disappearance of the platelets from the circulation after injecting glycogen was not found.

In the two experiments where a thrombocytopenia was produced (count less than 10,000/cu.mm.), the effect of protamine on the blood pressure was largely abolished. These were the only cases, in a large series of dogs, in which the intravenous injection of 10 mg./kg. of protamine in ten seconds did not cause the typical fall in blood pressure. This suggests that the thrombocytopenia produced by protamine is an important factor in its hypotensive action.

Discussion

It is evident from the work of Vartiainen and Marble, Shelley, Hodgkins, and Visscher, and others, that protamine is toxic to rats, guinea-pigs, and mice with an intravenous LD50 about 100 mg./kg. Smaller doses had relatively little effect, and Shelley, Hodgkins, and Visscher explained the effect as due to embolic vascular phenomena since protamine in these concentrations on addition to whole blood produced a thready precipitate and a gross agglutination of the blood corpuscles, related to the precipitation of fibrinogen by protamine, observed by Mylon, Winternitz, and de Suto-Nagy (1942). It seems probable that this action of protamine is responsible for the toxicity of protamine in the guinea-pig and other species, but it is unlikely that this is responsible for the toxicity in the dog. Mylon, Winternitz, and de Suto-Nagy state that it required 1.5 mg. of protamine per ml. of plasma to precipitate 80 per cent of the total fibrinogen and 0.5 mg. per ml. to affect the fibrinogen as judged by lengthening of the clotting time. A concentration of 0.05–0.10 mg. per ml., equivalent to the doses injected (5–10 mg. per kg.), fails to give a precipitate with oxalated plasma.

Not only is the action of protamine in the dog distinguished by its much lower dose level, but it is also differentiated by the nature of the toxic reaction. The chief effect in the dog is a temporary fall in blood pressure. This reaction depends markedly on the nature of the injection. It is produced only by a relatively rapid intravenous injection of protamine, and an effective fall in blood pressure provokes a tachyphylaxis lasting for some hours.

An attempt has been made to elucidate the nature of the hypotensive action of protamine. Fundamentally, the effect is due to vasodilatation of arterioles. This is shown by the simple but consistent observation that a venous sample taken when the blood pressure is falling is bright red and arterial in colour. Also, electrocardiographic tracings of a dog showed no change during the injection of salmine. Since the effect is still observed in the eviscerated animal, the liver and splanchnic area do not appear to take a great part in the action of protamine, but rather the muscular vessels are chiefly involved, as indicated by Thompson. The action of protamine in increasing capillary permeability through displacement of platelets and serum proteins discovered by Danielli (1940) is not a factor in the present experiments since haematocrit determinations did not show any immediate change. A very slight haemoconcentration occurred five minutes after the injection.

The vasodilatation and resulting fall in blood pressure after protamine might be due (1) to a direct effect on smooth muscle, or (2) to the liberation of histamine or other similar agents, or (3) to a reflex due to stimulation either of chemoreceptors, etc., in

the circulation or of the vasomotor centres. As indicated above, protamine possesses no histamine-like action on guinea-pig smooth muscle, and likewise it was not found to have any significant effect on the isolated dog ileum. This suggests that the action is not a direct effect on smooth muscle. The results on the spinal animals indicate that the action of protamine is not a simple one, but that, while the sharp initial fall in blood pressure may be due to a reflex vasodilatation, this is accompanied by a more slowly acting hypotension, suggestive of the release of pharmacologically active substances.

Since several protein products, simple proteins, and organic bases produce anaphylactoid shock in the dog by causing the liberation of histamine, one would expect a basic protein like protamine to have this action. Although we failed to detect histamine in the blood during protamine shock in the dog, the shock is relatively mild, and hence the amounts of histamine liberated may be quite small. The fact that the shock, on occasion, was modified by the previous administration of benadryl suggests that histamine release does occur and is a factor in the shock produced by protamine.

It is evident from the results obtained that associated with the effect of protamine on blood pressure in the dog is a marked thrombocytopenic effect. The very great fall in platelet count to 15 per cent of normal occurred only with the fall in blood pressure. Further, in contrast to leucocytes, the platelets were clumped and lysed by protamine directly. The production of a thrombocytopenia by the injection of glycogen prevented the depressor action of protamine, whereas failure to produce a thrombocytopenia with the same dose of glycogen resulted in failure to neutralize the action of protamine. This suggests that the action of protamine on the platelets plays an important role in the hypotensive effect of this substance. Possible mechanisms whereby platelet changes may cause a fall in blood pressure are suggested by the reports of Mylon, Winternitz, Katzenstein, and de Suto-Nagy (1942) and Rocha e Silva and Texeira (1946). Mylon *et al.* reported that thromboplastic shock could be prevented by cord section, which suggests that platelet changes could be responsible for that fraction of the depressor action of protamine prevented by cord section. On the other hand, Rocha e Silva and Texeira have suggested that in peptone shock platelet agglutination is responsible for the liberation of histamine. However, it should be pointed out that the thrombocytopenia may be a phenomenon accompanying the fall in blood pressure, rather than the cause of the hypotension, as the only experiments which provide evidence of a causal relationship are the few experiments in which glycogen was injected.

Rocha e Silva and Texeira (1946) have postulated that in peptone shock platelet disintegration leads to activation of plasma protease or fibrinolysin and that this causes the liberation of histamine. Since protamine can be used to demonstrate the presence of active fibrinolysin in blood, owing to its inhibition of the natural protease inhibitor (Scroggie, Jaques, and Rocha e Silva, 1947) this action of protamine might be responsible for its toxicity. Blood samples were taken from several animals and inspected for fibrinolysis, both by examining the blood directly and also by conducting the tests of Rocha e Silva and Texeira (1946) and MacFarlane (1946). No marked increase in fibrinolysis was observed after the injection of protamine. Activation of fibrinolysin may be a factor in the toxicity of protamine, but no evidence of this was seen in the present experiments, while in the experiments of Rocha e Silva and Texeira (1946), and of Mylon, Winternitz, and de Suto-Nagy (1942), the concentration of protamine used for fibrinolysis was much higher than the dosages used above.

It was observed that the effect of protamine on the platelets resulted in poor haemostasis as the animal bled freely from venipuncture wounds after the injection. This did not appear to be associated solely with the thrombocytopenia, as it appeared to persist even after the platelet count had risen again. Minor changes in the prothrombin time, referable to the effect on platelets and fibrinogen, were observed after protamine.

In addition to the effects on blood pressure in the dog, the protamines also affect respiration and the gastro-intestinal tract. These effects were minimal in the present study, and a separation of the respiratory and depressor effects of salmine was indicated with repeated injections. Thompson (1900) observed much greater toxicity when using clupein. The thymus histone studied previously gave results more closely resembling those of Thompson both in the effect on respiration and the duration of hypotension. In this connexion, it is of interest that the results reported by Jaques, Charles, and Best show that a fall in blood pressure due to the thymus histone did not produce refractoriness to salmine. Hence, while the various protamines and related basic proteins give similar pharmacological reactions, they are by no means interchangeable in this respect. This suggests that it is advisable to refer to them by their specific names—salmine, clupein, scombrin, etc., rather than by the generic name of protamine. It is interesting, in view of the effect of cord section on the depressor action, to note that the gastro-

intestinal symptoms reported by Jaques, Charles, and Best have been reported only in the unanaesthetized animal.

Since many of the symptoms resulting from the injection of protamine in the dog resemble a mild anaphylactic shock, this may be a heterophil anaphylaxis, like the sensitivity to *Ascaris suis* reported by Rocha e Silva, Grana, and Porto (1945). Skin tests and precipitin tests for protamine in dogs were conducted but were negative. Likewise, attempts to develop antibodies in guinea-pigs were negative. This agrees with reports in the literature that protamine is non-antigenic.

It is evident from the above that among the common experimental animals protamine is most toxic to the dog. It appears that protamine has been given as an intravenous injection clinically on occasion, and up to the present no alarming symptoms have been reported. The effect of protamine on human platelets has been tested by us, using the same technique as in Table VIII. There was a definite decrease in the platelet count (150,000 to 85,000 in ten minutes), but it was much less than the corresponding decrease in the dog. It is therefore unlikely that the toxicity of protamine will be of significance in its clinical use. The finding that no toxic effects resulted in the dog if the protamine were injected slowly suggests an additional reasonable precaution.

SUMMARY

1. The protamine, salmine, when rapidly injected intravenously was toxic to the dog in doses of 5 mg. per kg. At this dosage it showed little toxicity on intravenous injection in cats, rabbits, and guinea-pigs.

2. The intravenous injection of salmine in the dog caused a pronounced fall of blood pressure with rapid recovery and a slight apnoea. A second injection within six hours was without effect. A slow rate of injection did not cause a fall in blood pressure nor produce tachyphylaxis to a subsequent rapid injection. This effect of salmine on blood pressure was not changed by previous administration of atropine or of heparin, or by removal of the abdominal viscera. It was modified by the previous administration of benadryl, and by section of the spinal cord, and prevented by the previous intravenous injection of glycogen, when accompanied by thrombocytopenia. The fall in blood pressure was the result of extensive vasodilatation in the arterioles of muscles.

3. No increase in the amount of blood histamine or blood heparin could be detected after the injection of salmine. A definite transient thrombocytopenia and leucopenia occurred. Severe thrombocytopenia was associated with the fall in blood pressure, but leucopenia and a moderate fall in the platelet count occurred after the injection of the protamine in the absence of the fall in blood pressure.

4. Salmine added to blood in silicone-treated vessels caused thrombocytopenia but not leucopenia.

The author is greatly indebted to Professor C. H. Best and Dr. E. Fidlar, of the University of Toronto, for their interest in the problem. An active part in certain phases of the investigation was taken by Miss Mary Stewart, Miss A. E. Scroggie, Miss J. G. Cooper, and Miss Erica Lepp, and portions of this study were presented by the first three students as a part of the requirement for the honours degree in physiology and biochemistry at the University of Toronto. He is indebted to Mr. F. C. Monkhouse for the blood heparin determinations, and to Dr. A. M. Fisher, of the Connaught Medical Research Laboratories, for the protamine. The investigation was assisted by grants from the John and Mary R. Markle Foundation to the University of Toronto and from the National Research Council of Canada to the University of Saskatchewan.

REFERENCES

Ahlstrom, L., and von Euler, H. (1946). *Ark. kemi Min. Geol.*, **23A**, 1.
Anrep, G. V., Barsoum, G. S., and Ibrahim, A. (1947). *J. Physiol.*, **106**, 379.
Chargaff, E., and Olsen, K. (1937). *J. biol. Chem.*, **122**, 153.
Code, C. F. (1937). *J. Physiol.*, **89**, 237.
Danielli, J. F. (1940). *J. Physiol.*, **98**, 109.
De Cuyper, T. (1946). *Arch. int. Pharmacodyn.*, **72**, 360.
Fidlar, E., and Waters, E. T. (1941). *J. exp. Med.*, **73**, 299.
Hagedorn, H. C. (1938). *Skand. Arch. Physiol.*, **80**, 156.
Hein, F. (1943). *Arch. exp. Path. Pharmak.*, **202**, 228.
Jappelli, A. (1933). *Boll. Soc. ital. Biol. sper.*, **8**, 778.
Jaques, L. B., Charles, A. F., and Best, C. H. (1938). *Acta med. scand.*, Supp., **90**, 190.
Jaques, L. B., Fidlar, E., Felsted, E. T., and Macdonald, A. G. (1946). *Canad. med. Ass. J.*, **55**, 26.
Jaques, L. B., Monkhouse, F. C., and Stewart, M. (1949). *J. Physiol.* In press.
Jorpes, E., Edman, P., and Thaning, T. (1939). *Lancet*, **2**, 975.
MacFarlane, R. G. (1946). *Lancet*, **2**, 562.
Markowitz, J. (1937). *Textbook of Experimental Surgery*, p. 491, Baltimore: Wood.
Mendel, B., and Rudney, H. (1944). *Science*, **100**, 499.
Mylon, E., Winternitz, M. C., Katzenstein, R., and de Suto-Nagy, G. J. (1942). *Amer. J. Physiol.*, **137**, 280.
Mylon, E., Winternitz, M. C., and de Suto-Nagy, G. J. (1942). *J. biol. Chem.*, **143**, 21.
Reiner, L., de Beer, E. J., and Green, M. (1942). *Proc. Soc. exp. Biol., N.Y.*, **50**, 70.
Rocha e Silva, M., Grana, A., and Porto, A. (1945). *Proc. Soc. exp. Biol., N.Y.*, **59**, 57.
Rocha e Silva, M., and Texeira, R. M. (1946). *Proc. Soc. exp. Biol., N.Y.*, **66**, 326.
Scroggie, A. E., and Jaques, L. B. (1948). Unpublished.
Scroggie, A. E., Jaques, L. B., and Rocha e Silva, M. (1947). *Proc. Soc. exp. Biol., N.Y.*, **66**, 326.
Shelley, W. B., Hodgkins, M. P., and Visscher, M. B. (1942). *Proc. Soc. exp. Biol., N.Y.*, **50**, 300.
Thompson, W. H. (1900). *Hoppe-Seyl. Z.*, **29**, 1.
Vartiainen, I., and Marble, A. (1941). *J. Lab. clin. Med.*, **26**, 1416.
Wells, J. A., Morris, H. C., Bull, H. B., and Dragstedt, C. A. (1945). *J. Pharmacol.*, **85**, 122.