HEPARINEMIA (?)*

An Anticoagulant in the Blood of Dogs with Hemorrhagic Tendency after Total Body Exposure to Roentgen Rays

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Plates 1 and 2

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Hemorrhage is one of the important abnormalities occurring in animals and man exposed to large doses of ionizing radiation delivered over the entire body. Fernau, Schrank, and Zarsicki (1) as early as 1913, reported hemorrhage in rabbits following the injection of one hundred or more electrostatic units of polonium. Death usually occurred before the 12th day, at which time the blood was incoagulable. These observations have been confirmed by Falta (2) and others (3, 4). In these reports, hemorrhages were extensive and all organs of the body were affected. It was suggested that the associated thrombocytopenia was the probable cause of the hemorrhagic state.

In the dog exposed to x-irradiation of the entire body many pathologic alterations may occur. The most obvious of these are hemorrhage and infection. Hemorrhage is capable in itself of killing the animal, and it may appear as a result of exposure to most forms of ionizing irradiation (1–6).

The Irradiation Syndrome in Dogs

The data comprising this report were obtained from studies on dogs given an x-ray exposure of 450 roentgen units over the entire body. Daily observations were made on each animal whenever possible, including the whole blood clotting times, the prothrombin time, clot retraction, erythrocyte count, leukocyte count, differential leukocyte count, platelet count, hematocrit reading, and sedimentation rate. Daily recordings of the rectal temperatures were made and the physical condition of each animal was noted. The earliest signs of bleeding were usually observed at the points of greatest trauma, especially at the point of needle puncture. Hemorrhages in the mouth, the soles of the feet, and the subcutaneous tissue on the sides of the animal usually appeared first, generally during the 2nd week. The hemorrhagic state progressed and there were spontaneous hemorrhages from the rectum, vagina, and urinary tract. At death, 2 or 3 days later, hemorrhages were the chief findings. They were abundant throughout the gastrointestinal tract and were most marked in the colon and proximal four-fifths of the stomach. They occurred throughout the small bowel but were more prominent in the duodenum and lower ileum. They were almost invariably present in

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the heart muscle where blood was extravasated along the course of the coronary vessels. The lungs, parietal pleura, and diaphragm frequently showed extensive subserous hemorrhages, and they were occasionally seen in the gall bladder, urinary bladder, and skeletal muscles. The lymph nodes throughout the entire body appeared swollen, hemorrhagic, and soft. Although gross evidence of hemorrhage was not generally present in the solid organs, microscopic evidence of hemorrhage was almost always found. In general those organs subject to motion displayed the most marked evidence of hemorrhage.

TABLE I

The average clotting time, hemocytologic findings, hematocrit reading, and sedimentation rates are recorded for 25 dogs, exposed to 450 r over the entire body and untreated thereafter. The findings as given were complicated by occasional blood transfusions.

Days	Clotting time	Platelet count	Blood counts		Hemoglobin	Hematocrit reading	Sedimentation rate (60 min.)	Smear					Body temperature	Day of first hemorrhage	Death
			RBC	WBC				Neutrophiles	Lymphocytes	Monocuclears	Eosinophiles	Basophiles			
	min.	*c.mm.*	*c.mm.*	*c.mm.*	*gm. per cent*	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*°F.*		
Control	11.7	300,000	5.94	12,432	14.6	48	7.06	57.375	39.75	.05	2.1250	0.125	102.0		
1st	18	247,000	5.47	8,182	13.8	46.8	10.7	69.2	27	1.4	2.4	0	101.85		
2nd	17	211,200	6.13	5,760	13.7	44	19.8	75.6	20.4	0.6	2.4	0	101.85		
3rd	29	210,400	6.07	6,892	14.4	45	12.1	78.67	17.083	0.083	4.083	0.083	101.9		
4th	28	114,800	5.23	3,000	12.8	42.4	14.6	51.17	43	0.33	0.17	0	101.73	2 dogs	
5th	36	122,500	5.08	2,265	12.0	37.9	12.7	48.25	43.675	0.75	0.125	0	102.2	3 "	
6th	30	133,111	5.124	1,484	12.1	38.6	27.75	40.7	57.7	0.8	0.8	0	103.0	4 "	2 dogs
7th	35	111,583	4.74	504	10.7	36.0	26.2	30.4	57.6	2.0	0	0	103.2	4 "	1 "
8th	36	92,145	4.93	461	10.9	35.2	47.0	23.4	75.4	1.1	0	0.1	103.7	3 "	1 "
9th	49.49	78,500	4.316	702.9	13.37	33.4	47.64	26	74	0	0	0	104.6		3 "
10th	33.3	91,700	3.94	175	9.45	30.5	56.5	15	84	1	0	0	105.6	3 "	4 "
11th	41.15	52,923	3.566	292.45	9.84	31.2	59.5	10	90	—	—	—	104.98	3 "	3 "
12th	—	—	—	—	—	—	—	—	—	—	—	—			5 "
13th	81.7	34,167	—	433.3	10.13	—	—	—	—	—	—	—			
14th	—	—	—	—	—	—	—	—	—	—	—	—		3 "	2 "
15th	93.3	20,000	—	—	—	—	—	—	—	—	—	—			4 "

Many animals displayed bleeding ulcers in the mouth, duodenum, and especially in the colon. These hemorrhages were frequently complicated by infection.

The remainder of the picture displayed by dogs was that of an acute intoxication. They became listless and failed to eat. Fever developed, the sedimentation rate was increased, leucopenia and thrombocytopenia appeared, and frequently bacteria could be found in the terminal blood smear. Death usually occurred between the 9th and the 12th days. The hemorrhagic state was characterized by a prolonged clotting time which was easily studied.

The picture displayed by these untreated dogs was almost uniform, although the survival times varied from 7 to 15 days with an average of 11 days. The hematologic findings and clotting results for all untreated animals were averaged and are presented in Table I. To shorten this table, some data have been omitted.

EXPERIMENTAL

The technique used in the exposure was fairly constant. The source of x-rays was a 200 K.V.P. machine. The rays were filtered with 0.5 mm. of copper and 1 mm. of aluminum. The tube was operated at 15 milliamperes. The dosage rate was 6 r per minute as measured in air at a distance correspondng to the center of the animal's body and was checked before each exposure. This method has been described by Hagen and Zirkle (7).

The clotting studies made included the prothrombin time (8), the whole blood clotting time (9), and a heparin titration test carried out on whole blood. This last procedure will be described later.

Because the course and laboratory findings displayed by the first animal, dog 1-08, did much to pattern the subsequent experiments, the observations made on this animal are presented as representative of the group.

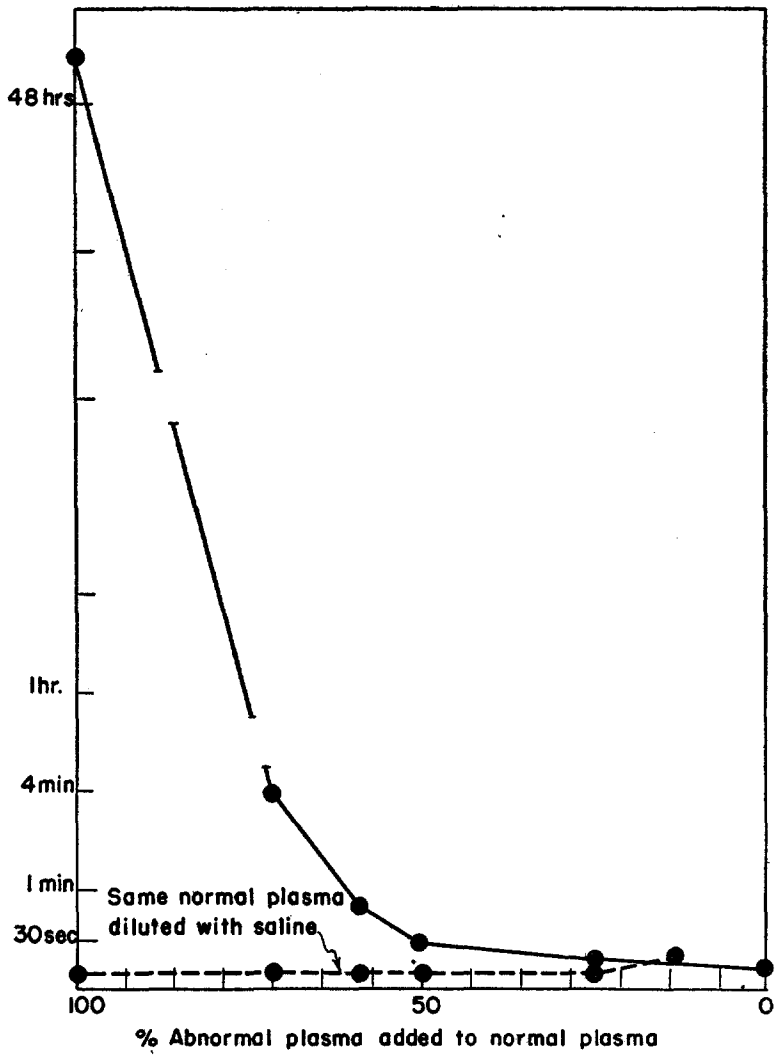
Dog 1-08, male, weighing 9.7 kilos remained in apparent good health for the first 10 days after x-ray exposure. The clotting time, however, was markedly prolonged after the 6th day, and on the 9th day there was bleeding from the mucous membranes of the mouth and rectum. The bleeding time was also prolonged and the animal bled freely from all points of needle puncture even though some of these punctures had been made several days previously. On the 9th day the dog's blood failed to clot and remained fluid in the test tube even after it became hemolyzed 2 days later. It could not be made to clot by adding thromboplastin, so the ordinary prothrombin time could not be determined. The platelet count on the 9th day was 260,000 and therefore did not seem to account for the bleeding. The blood did clot, however, when trypsin, papain, or purified thrombin was added. Since prothrombin is necessary for trypsin to clot fibrinogen, and since papain or thrombin clot fibrinogen independent of prothrombin, these findings demonstrated that both prothrombin and fibrinogen were present.

(*a*) *Demonstration of an Anticoagulant.*—

The failure of the blood to clot except when the above mentioned substances were added, even though thromboplastin, fibrinogen, prothrombin, and calcium were present, suggested that its incoagulable state might be due to the presence of an anticoagulant. This phase of the problem was next explored.

The plasma of dog 1-08 was mixed at various concentrations with oxalated normal dog plasma. The clotting times of these mixtures were then determined after the addition of thromboplastin and calcium chloride. Under these conditions the clotting times of the normal plasma were prolonged. For example, when the mixture contained 25 per cent normal plasma and 75 per cent plasma from dog 1-08, the clotting time was 4 minutes. In contrast, when a mixture of 25 per cent normal plasma and 75 per cent normal saline was used, the clotting time was 20 seconds. The observations were repeated, using separately various amounts of abnormal plasma (dog 1-08) and similar quantities of saline, and the effects of each upon the clotting times of normal plasma observed. The results are shown graphically in Text-fig. 1. These findings indicated that the delayed clotting times of the normal plasma-abnormal plasma mixtures were due to an active inhibition, not to mere dilution of

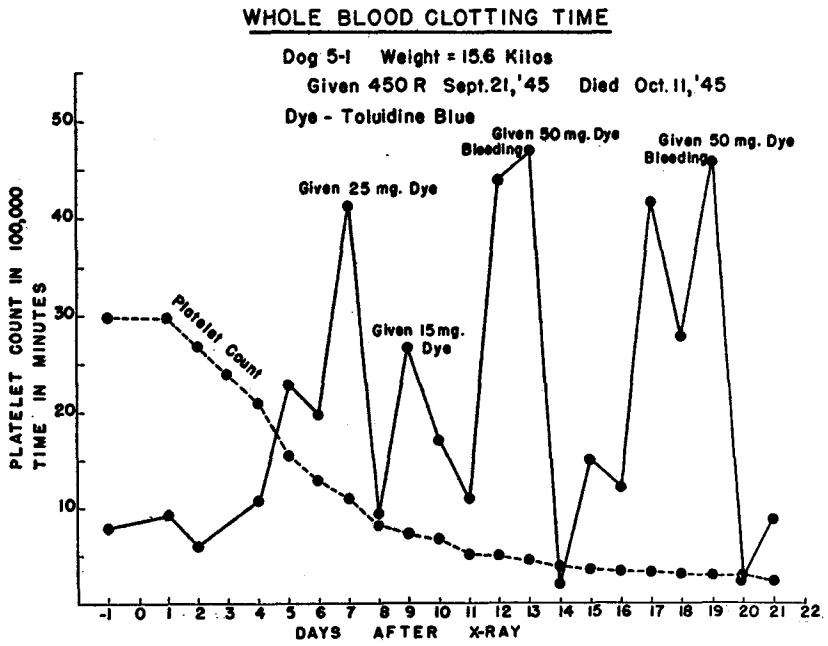
normal plasma by an inactive plasma. It was presumed that the abnormal plasma contained an anticoagulant.



TEXT-FIG. 1. The inhibitory effect of the plasma of dog receiving 450 r on the clotting of normal plasma.

In view of these findings, it seemed worth while to examine the abnormal plasma for the presence of heparin, the only known, naturally occurring endogenous mammalian anticoagulant. Heparin, an acid substance, can be bound and biologicaly inactivated by certain basic dyes and proteins (10, 11). Protamine sulfate and

toluidine blue are both active in this respect and can be used to exclude heparin from the clotting system by the addition of just enough of either to overcome the anticoagulant properties of heparin. These tests were made with the plasma of dog 1-08. Standard volumes of oxalated plasma with various amounts of toluidine blue or protamine sulfate were incubated for 20 minutes, and then beef lung thromboplastin and calcium chloride were added and the clotting times determined. Both of these substances proved capable of returning the clotting time to normal from its previously



TEXT-FIG 2. Demonstration of the effect of toluidine blue to return the prolonged clotting time to normal. The curve is characteristic of the phenomenon in other dogs which had received 450 r.

incoagulable state. The results are recorded in Text-fig. 2. The results of these two experiments suggest that some of the biochemical properties of the anticoagulant were similar to those of heparin.

The amount of toluidine blue necessary to restore the normal clotting time in dog 1-08 was calculated on the basis of these *in vitro* studies, after estimating the plasma volume; and this amount (25 mg.) was injected intravenously. Within 20 minutes all external bleeding stopped. Blood drawn at this time clotted within 4 minutes, while three separate samples, each distributed in a series of ten tubes, drawn just before injection remained fluid until they were discarded 2 days later. The blood from the dog was sampled on five more occasions during the 12 hour period following the injection, and on each occasion the clotting time was normal. Subsequently, it was observed

that gastrointestinal hemorrhages were always present in the untreated animals, and that these could also be prevented by the administration of toluidine blue or protamine sulfate (Figs. 1 and 2).

Both toluidine blue and protamine sulfate proved anticoagulant in themselves, when used in excess. Their anticoagulant properties are shown in Tables II and III. However, the amounts necessary to prevent clotting were considerably more than that required to establish normal clotting in dog 1-08, as was also the case in other dogs subsequently tested.

TABLE II

Effect of Various Quantities of Toluidine Blue on the "Prothrombin Time" of the Plasma of Dog 1-08

Toluidine blue	"Prothrombin time"	Protamine sulfate	"Prothrombin time"
mg./0.2 cc. of plasma	*sec.*	*mg./0.2 cc. of plasma*	*sec.*
0.00	Incoagulable	0.00	Incoagulable
0.020	110	0.020	100
0.040	85	0.040	65
0.060	70	0.060	60
0.080	300	0.080	480
0.160	12 hrs.	0.160	12 hrs. approximately
0.225	20 "	0.225	20 " "

TABLE III

The Anticoagulant Effect of Toluidine Blue

Toluidine blue	"Prothrombin time"
mg. per cc. of plasma	*sec.*
0.02	28
0.018	26
0.016	24
0.014	23
0.012	23
0.010	22
0.008	20
0.006	20
0.004	19
0.002	20
0.000	20

(*b*) *Observations on Other Factors Concerned with Coagulation.*—Once it was possible to cause the plasma to clot, examinations for other plasma or blood deficiencies relevant to coagulation were made. The factors examined included: plasma prothrombn, serum calcium, plasma fibrinogen, and the platelets.

1. Plasma Prothrombin: The prothrombin time was determined on the plasma of all the irradiated dogs. Except in the terminal state, it was found normal if the plasma was first incubated with appropriate amounts of toluidine blue or protamine sulfate. The latter proved more uniformly active, and save for the first few experiments, protamine sulfate was the agent used. When plasma was not first incubated with protamine sulfate, the "prothrombin time" was occasionally prolonged by the anticoagulant present.

Vitamin K was administered to a group of irradiated dogs and was found to be ineffective in preventing hemorrhage. Data from four of these dogs are presented in Table IV. These animals received intravenously 5 mg. per day of an active vitamin K preparation (synkayvite-Roche) throughout the post-irradiation period. Three of these animals showed the usual prolonged clotting times which appeared 1 to 2 days before death. At autopsy hemorrhages were widespread, and there was no evidence that vitamin K was of therapeutic value.

The fourth dog (No. 1-8) received, in addition to vitamin K, a single 200 mg dose of dicumarol 24 hours after irradiation. The effect of this drug was of interest in that a recovery of prothrombic activity did take place before the animal died. It will also be noted that while the prothrombin time recovered in this animal, the whole blood clotting time remained prolonged.

Both toluidine blue and protamine sulfate proved ineffective in overcoming the prolonged prothrombin time induced by dicumarol administration to normal dogs. Six animals were given dicumarol for 2 days and when the prothrombin time was prolonged, toluidine blue was administered to four dogs and protamine sulfate was given to the remaining two animals (Table V). Five of the six dogs died on the 5th and 6th days from massive hemorrhage even though 3 to 5 mg. per kilo of body weight of these substances were administered daily for several days. The failure of these agents to influence the prothrombic activity under these conditions was not surprising. A fact of interest was that the prothrombin reduction in dogs 2-7 and 2-1, was not associated with a marked increase of the whole blood clotting time until after a reduction to less than 20 per cent of normal had occurred. The extensive hemorrhages observed at postmortem seemed out of proportion to the prolongation in the whole blood clotting time, an observtaion emphasizing the fact that the prothrombin time and whole blood clotting times are different expressions of hemorrhagic states and may on occasion be indices to different and unrelated phenomena.

2. Serum Calcium: Although there is no known spontaneous hemorrhagic state attributed to a deficiency of the calcium ion or known to be corrected by the administration of calcium salts, a study of the calcium, phosphorus, and magnesium ions in the irradiated animal was undertaken as a means of exploring all the theoretical possibilities of prolonging the whole blood clotting times. The results of these studies are reported in Table VI. Daily studies

TABLE IV

Failure of Vitamin K to Prevent Hemorrhages in Irradiated Dogs

Dog No. and weight		No. of days after radiation												
		0	1	2	3	4	5	6	7	8	9	10	11	12
8-60	Whole blood clotting time, *min.*	22	11	1—	10	12	23	10	11	8	—	16	53	
	Prothrombin time, *per cent.*	100	100	—	100	100	100	100	100	100	—	100	74	
19.0 kg.	Platelet count, *per c.mm.*	260,000	290,000	—	190,000	130,000	130,000	88,000	96,000	130,000	—	50,000	48,000	
	Vitamin K given, *mg.*	—	5	—	5	5	10*	10*	10*	10*	—	10*	10*	
	Toluidine blue given, *mg.*	—	—	—	—	—	—	—	—	—	—	—	Bleeding	Dead
2-6	Whole blood clotting time, *min.*	18	14	—	10	24	14	12	9	21	—	18	66	Bleeding
	Prothrombin time, *per cent.*	100	100	—	100	100	100	100	100	100	—	100	100	stopped after dye
17.0 kg.	Platelet count, *per c.mm.*	180,000	220,000	—	230,000	140,000	120,000	112,000	120,000	80,000	—	80,000	50,000	injection
	Vitamin K given, *mg.*	—	5	—	5	5	10*	10*	10*	10*	—	10*	10*	
	Toluidine blue given, *mg.*	—	—	—	—	—	—	—	—	—	—	—	60	Dead
2-2	Whole blood clotting time, *min.*	9	8	21	16	30	11	18	—	8	24	47	85	
	Prothrombin time, *per cent.*	100	100	84	—	100	100	100	—	100	100	85	95	
7.5 kg.	Platelet count, *per c.mm.*	220,000	190,000	220,000	220,000	120,000	120,000	105,000	—	104,000	64,000	64,000	50,000	
	Vitamin K given, *mg.*	—	5	5	10*	10*	10*	10*	—	10*	10*	—	10*	
	Toluidine blue given, *mg.*	—	—	—	—	—	—	—	—	—	—	—	Bleeding	Dead
1-8	Whole blood clotting time, *min.*	24	18	28	35	61	30	71	—	70	65	165		
	Prothrombin time, *per cent.*	100	100	42	26	30	7	13	—	10	42	77		
7.5 kg.	Platelet count, *per c.mm.*	340,000	220,000	280,000	340,000	136,000	120,000	120,000	—	64,000	80,000	48,000		
	Vitamin K given, *per c.mm.*	—	5	5	10*	10*	10*	10*	—	10*	10*	—		
	Toluidine blue given, *mg.*	—	—	—	—	—	—	—	—	—	—	—		
	Dicumarol given, *mg.*	—	200	—	—	—	—	—	—	— (Given 150 cc. of blood)	—	Bleeding		Dead

* Intravenous administration.

TABLE V

Failure of Toluidine Blue or Protamine Sulfate to Correct Hemorrhage Consequent on Administration of Dicumarol

Dog No. and weight		Control before therapy	No. of days 1	2	3	4	5	6	7	8	9	10	
2-7	Prothrombin time, *per cent*	88	—	25	12	13	*Remarks:* Died on 5th day. Developed distemper on 2nd day and massive hematoma formed in right thigh on 3rd day.						
21.0 kg.	Whole blood clotting time, *min.*	20	—	19	23	22							
	Dicumarol given, *mg.*	300	—	200	—	—	42						
	Platelet count *per c.mm.*	190,000	—	180,000	170,000	135,000							
	Toluidine blue given, *mg.*	—	—	—	50	50							
2-1	Prothrombin time, *per cent*	100	—	28	5	12	16						
	Whole blood clotting time, *min.*	16	—	22	90	45	55	*Remarks:* Dog died on 6th day from massive hemorrhage.					
8.0 kg.	Platelet count, *per c.mm.*	260,000	—	270,000	190,000	340,000	460,000						
	Dicumarol given, *mg.*	200	—	200	—	—	—						
	Toluidine blue given, *mg.*	—	—	—	50	50	—						
1-9	Prothrombin time, *per cent*	100	—	48	15	5	*Remarks:* Developed a large hematoma on 3rd day in right thigh. Hematocrit reading on 3rd day was 54 and it fell to 41 on the 4th day. Died on 5th day.						
	Whole blood clotting time, *min.*	28	—	25	36	85							
6.9 kg.	Platelet count, *per c.mm.*	240,000	—	240,000	200,000	190,000							
	Dicumarol given, *mg.*	200	—	150	—	—							
	Toluidine blue given, *mg.*	—	—	—	50	50							
1-7	Prothrombin time, *per cent*	100	—	40	27	20	60	83	100		100	100	
	Whole blood clotting time, *min.*	17	—	—	50	45	35	15	21		16	24	
9.0 kg.	Platelet count, *per c.mm.*	290,000	—	250,000	300,000	400,000	480,000	490,000	460,000		—	—	
	Dicumarol given, *mg.*	200	—	200	—	—	—	—	—		—	—	
	Toluidine blue given, *mg.*	—	—	—	50	50					—	—	*Remarks: Dog survived.*
6-3	Prothrombin time, *per cent*	100	—	28	10	8	*Remarks:* Developed large retroperitoneal hematoma and died on 5th day.						
10.6 kg.	Whole blood clotting time, *min.*	21	—	26	31	29							
	Dicumarol given, *mg.*	200	—	200	—	—							
	Platelet count, *per c.mm.*	290,000	—	260,000	270,000	290,000							
	Protamine sulfate, *mg.*	—	—	—	50	50							
6-4	Prothrombin time, *per cent*	100	—	34	13	10	6	*Remarks:* Died on 6th day from massive hemorrhage.					
9.7 kg.	Whole blood clotting time, *min.*	18	—	19	26	30	35						
	Dicumarol given, *mg.*	200	—	200	—	—	—						
	Platelet count, *per c.mm.*	260,000	—	270,000	240,000	270,000	260,000						
	Protamine sulfate, *mg.*	—	—	50	50	50							

were not carried out on all animals, although in no case was there more than a 3 day period between determinations. The figures presented are averages based on the individual results obtained on all animals tested on a given day. The serum calcium was determined by the method of Cramer and Tisdell (12), the serum phosphorus by the Bodansky procedure (13), and the serum magnesium by Hoffman's method (14). No significant deviations from the normal values were observed, although the tendency to hemorrhage progressed until

TABLE VI

Average Values of Serum Calcium, Phosphorus, and Magnesium in Irradiated Dogs

Day	No. of dogs tested	Serum			Clotting time	Platelet count	Final outcome
		Calcium	Phosphorus	Magnesium			
		mg. per cent	*mg. per cent*	*mg. per cent*	*min.*	*per c. mm.*	
Control	13*	11.4	3.7	2.0	17	340,000	
1st	5	10.4	3.8	2.8	10	299,000	
2nd	6	11.1	3.6	2.2	14	207,000	
3rd	10	10.8	4.1	2.2	29	197,000	
4th	8	10.2	3.9	2.2	19	149,000	
5th	6	10.5	3.5	2.1	21	130,000	
6th	7	10.2	4.3	2.2	19	114,000	One dog died
7th	9	9.7	3.5	2.2	33	133,000	One " "
8th	8	10.8	3.8	2.2	24	99,000	Three dogs "
9th	5	10.3	3.4	2.2	48	84,000	Two " "
10th	5	10.2	3.8	1.9	47	76,000	One dog "
11th	4	10.2	2.8	2.2	55	59,000	
12th	4	10.0	4.0	2.6	53	52,000	" " "
13th	2	10.5	3.7	2.6	35	44,000	Two dogs "
16th	2	10.7	4.5	2.1	31	45,000	Both dogs partially treated for hemorrhage

*Two control determinations were made on each animal during the week immediately preceding irradiation.

death. Four exceptions were noted in four dogs not included in Table VI. The serum calcium levels in these animals were reduced, but these changes occurred before hemorrhage developed. Four of the animals reported in Table VII were given calcium gluconate when hemorrhage appeared, but no improvement was noted.

3. Plasma Fibrinogen: The fibrinogen level was not determned. The fact that an ample clot formed indicated the presence of fibrinogen. Clot retraction, however, was retarded or completely absent and this defect was closely related to the platelet count.

There was some evidence that the clot itself was not entirely normal in the blood of the untreated irradiated dog. The blood appeared to gel before an

actual clot was formed. The gelled blood could be reverted to a fluid state if the blood was shaken before a solid clot had formed. Considerable time often elapsed after the gel appeared before firm clotting took place. Shreds of fibrin did not appear until shortly before the solid clot appeared. This phenomenon, however, was not studied extensively, though the observation was made that it occurred also in slightly heparinized normal dog blood in which coagulation was delayed. It was not observed in prothrombin-deficient dog blood or in human hemophilic blood in which the whole blood clotting time was prolonged. Once the clot began to form in these latter two conditions it was rapidly completed without visibly exhibiting any tendency to first gel.

4. Platelets: The total number of circulating platelets is reduced after marked exposure to x-irradiation, and aplasia or the marrow may result (5, 6). While the bleeding irradiated dogs of our series always developed a profound thrombocytopenia, this reduction of platelets did not always coincide with the onset of bleeding. In some animals bleeding preceded thrombocytopenia, in others it developed afterwards, but the majority showed the reduction of platelets at about the time hemorrhage appeared. This relation was not that of cause and effect, as was demonstrated in dog 1-08. In this animal the platelet count was 260,000 when hemorrhage began and the blood became incoagulable.

Once hemorrhage developed and the clotting time was prolonged, freshly drawn transfusions of citrated whole blood had little effect. Of all the measures employed, only toluidine blue or protamine sulfate proved effective. Certain other members of the thionin series (azure A, azure B, thionin, and to some extent methylene blue) were also tested and showed some antihemorrhagic effect, but, save for methylene blue, they proved too toxic for therapeutic use.

The lack of a cause and effect relation between the thrombocytopenia and the hemorrhagic state was also brought out by the fact that toluidine blue or protamine sulfate stopped the tendency to bleed but did not elevate the platelet count. In Table VII are presented data on nine dogs whose clotting times returned to normal after dye injecton without any rise in the platelet count. This same phenomenon was even better demonstrated in animals given repeated injections of either toluidine blue or protamine sulfate which controlled the hemorrhage even though the thrombocytopenia was marked. For example in dog 5-1 the clotting time was returned to normal on five occasions although the platelet count remained severely reduced from the seventh postirradiation day until death (Text-fig. 2).

It would appear that the thrombocytopenia associated with x-irradiation, in the dog at least, did not materially contribute to the prolonged clotting time. Just what may be the significance of the thrombocytopenia with reference to the hemorrhagic state in this condition remains to be seen. The thrombo-

TABLE VII

Effect of a Single Injection of Toluidine Blue on the Whole Blood Clotting Time in Irradiated Dogs with Hemorrhage

Dog. No.	Weight	Total amount of dye given	Whole blood clotting times			
			Before dye injection	24 hrs. after injection	48 hrs. after injection	72 hrs. after injection
	kg.	*mg.*				
1-03	11.2	10				
Whole blood clotting time, *min.*			84	11	90	—
Prothrombin time, *per cent*			100	100	100	
Platelet count, *per c.mm.*			20,000	15,000	15,000	
1-08	7.4	24				
Whole blood clotting time, *min.*			Incoagulable	4		
Prothrombin time, *per cent*			100	100		
Platelet count, *per c.mm.*			70,000	70,000		
3	11.0	50				
Whole blood clotting time, *min.*			40	26	15	
Prothrombin time, *per cent*			100	100	100	
Platelet count, *per c.mm.*			110,000	96,000	72,000	
9-45	11.2	50				
Whole blood clotting time, *min.*			56	20	36	
Prothrombin time, *per cent*			100	100	100	
Platelet count, *per c.mm.*			48,000	32,000	40,000	
1-0	10.6	75				
Whole blood clotting time, *min.*			35	4		
Prothrombin time, *per cent*			100	100		
Platelet count, *per c.mm.*			90,000	70,000		
5-1	15.0	80				
Whole blood clotting time, *min.*			51	2	1	14
Prothrombin time, *per cent*			100	100	100	100
Platelet count, *per c.mm.*						
7-4	15.5	80				
Whole blood clotting time, *min.*			120	4	6	32
Prothrombin time, *per cent*			100	100	100	100
Platelet count, *per c.mm.*			90,000	60,000	60,000	—
6-5	9.5	50				
Whole blood clotting time, *min.*			25	16	11	32
Prothrombin time, *per cent*			100	100	100	100
Platelet count, *per c.mm.*			200,000	200,000	200,000	86,000

TABLE VII—*Concluded*

Dog. No.	Weight	Total amount of dye given	Whole blood clotting times			
			Before dye injection	24 hrs. after injection	48 hrs. after injection	72 hrs. after injection
	kg.	*mg.*				
4-99		100				
Whole blood clotting time, *min.*			28	9	29	
Prothrombin time, *per cent*			100	100	100	
Platelet count, *per c.mm.*			160,000	150,000	140,000	
5		40				
Whole blood clotting time, *min.*			64	150	170	
Prothrombin time, *per cent*			8	5	2	
Platelet count, *per c.mm.*			96,000	100,000	80,000	

TABLE VIII

Results of the Attempted Isolation of Heparin from the Blood of Irradiated Dogs with Hemorrhage

Dog. No.	Volume blood	Whole blood clotting time on day of death	Heparin-like material obtained	Units per mg.
		min.	*mg.*	
9-5	475	87	11	110
9-6	860	65	9	100
9-7	320	68	6	110
9-8	430	47	4	90

cytopenia may well have been responsible for the impaired clot retraction and the prolonged bleeding time, neither of which responded to protamine sulfate or toluidine blue administration.

(*c*) *Attempts to Isolate Heparin.*—Reported below is our experience with attempts at heparin isolation from the blood of irradiated dogs with hemorrhage. The procedure of Jaques and Waters (15) was employed for this purpose. Efforts at isolating heparin from blood were complicated by the fact that in most animals the blood was not rendered entirely incoagulable but that instead the clotting time was prolonged to three to five times normal. This fact, coupled with the relatively inefficient method of heparin recovery, made this part of our problem especially difficult.

Four dogs which had received 450 r irradiation over the entire body were exsanguinated under local anesthesia with novocaine, and the bloods obtained were separately analyzed for the amorphous sodium acid salt of heparin. The resulting data are presented in Table VIII. The anticoagulant property of the amorphous material obtained in each case was high, and when toluidine blue or

protamine sulfate was added to it in solution, its anticoagulant property was lost. About 3 mg. of dye was necessary to inactivate 2 mg. of the anticoagulant. This same ratio held also for protamine sulfate. In the case of the dye a small metachromatic precipitate formed in the bottom of the test tube about 20 minutes after the dye had been added. The anticoagulant was heat-stable at 100°C. for 20 minutes.

The inefficiency of the method of isolation in our hands was demonstrated by the fact that in nine attempts at recovery of heparin from normal blood to which a standard sodium acid salt of heparin had been added, only 5 to 15 per cent of the original material was recaptured. The greatest loss appeared to take place in the initial procedure of protein precipitation.

DISCUSSION

The experimental observations here reported suggest that the defect responsible for the hemorrhagic state in the irradiated dogs was the presence of an anticoagulant in the blood. This substance so far as it was tested, resembled heparin.

Heparin has been obtained in high degrees of purity by many workers, yet it is still known mostly by its biologic properties. It is thought to be a polysaccharide with many sulfuric acid groups, and is classified as an ester of mucotin sulfuric acid, but its chemical identity is not yet known (11). Certain of its constituents, especially its sulfur content and its crystalline characteristics are at the moment under controversy. While the material isolated from the blood of our animals seemed in no way dissimilar from a standard sodium heparin salt, we can class it only as "heparin-like" because the identity of heparin itself is unknown.

Different lots of toluidine blue have varied considerably in their antiheparin activity *in vitro*. Many samples of toluidine blue showed no activity even though they were obtained from the same source. The intravenous administration of the dye, however, controlled bleeding even though the antiheparin activity was slight on *in vitro* titration, but a longer period of time was required to control bleeding in the case of the less active preparations. Titrations of heparin activity with protamine sulfate, however, were always satisfactory, and while some variations occurred from lot to lot, each new lot could readily be standardized against the standard heparin preparation.

It was not the purpose of these experiments to increase the survival periods of fatally irradiated dogs, although the data suggest that when hemorrhage was controlled life may have been prolonged; the administration of toluidine blue or protamine sulfate at fairly frequent intervals lengthened life by an average of 10 days. The dose of 450 r x-ray used was approximately 150 to 175 r greater than the LD_{50} for dogs (300 r) and resulted in the death of the untreated animal after 11 days on the average. At 450 r there were no survivals in either the

control group or the animals given toluidine blue or protamne sulfate. The cause of death in these animals was not determined. Infection in the terminal phase was always a prominent finding, but probably other factors were involved. It is known that in less severely injured animals (dogs and rabbits) the marrow will recover to a large extent, even though at the height of the disturbance the histologic evidence of injury may be just as great in the animal which survives as in that which dies (7). This being so, the control of hemorrhage in the less severely irradiated animals may contribute to the animal's recovery.

SUMMARY

When the entire body of dogs was exposed to 450 units of Roentgen irradiation a hemorrhagic syndrome developed which was characterized by thrombocytopenia, prolonged clotting and bleeding times, and neutropenia.

The prothrombin time remained normal until about 24 hours before death. The calcium, phosphorus, and magnesium levels were not altered. Fibrinogen was present but syneresis was poor.

Toluidine blue and protamine sulfate, substances which can inhibit the biologic action of heparin, restored the clotting time to normal.

The hemorrhagic state was not materially altered by transfusions, vitamin K, or vitamin C.

Toluidine blue and protamine sulfate were ineffective in the control of hemorrhage produced by dicumarol.

The defect responsible for bleeding after irradiation appeared to be the presence in the circulation of an anticoagulant whose properties, so far as tested, were indistinguishable from those of heparin.

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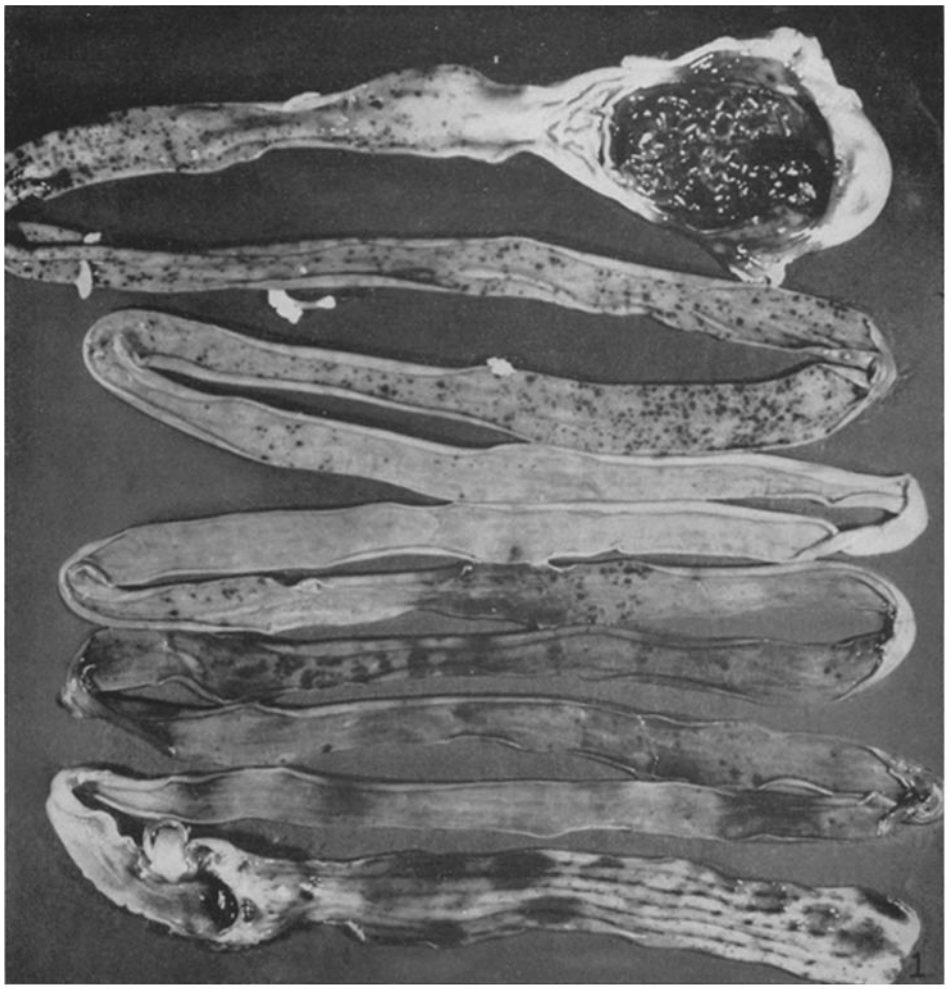
EXPLANATION OF PLATES

PLATE 1

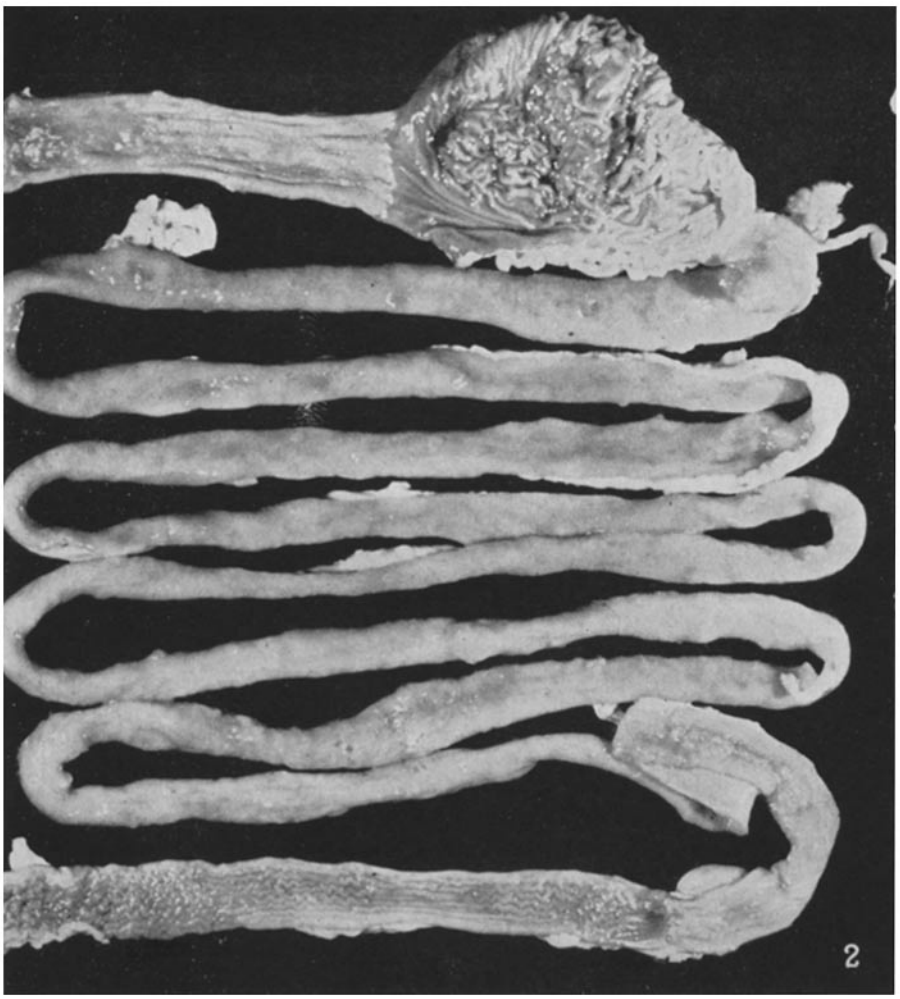
FIG. 1. Typical picture of gastrointestinal tract of untreated irradiated dog (450 r).



(Allen *et al.*: Heparinemia (?))

PLATE 2

FIG. 2. Gastrointestinal tract of irradiated dog (450 r) treated with frequent injections of toluidine blue. Note, however, that the edema and thickening of the gut wall persist.



(Allen *et al.*: Heparinemia (?))