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PROGRESS REPORT
ABNORMAL BLEEDING

J. Garrott Allen

March 1950

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FOREWORD

The following series of papers is presented in this supplement in order to maintain continuity insofar as possible with reference to the problem of blood coagulation as influenced by total-body X irradiation of the dog. These papers, some preliminary in nature, are presented in the spirit of a progress report. A complete study of the hemostatic mechanism has not been made. For example, this report contains no information of the status of the vascular walls which are probably seriously altered in the late stages of the irradiation syndrome.

The work was carried out under the auspices and cooperation of the Office of the Surgeon General of the U. S. Army, the Biological Division of the Argonne National Laboratory, and the Department of Surgery of the University of Chicago. The authors wish to acknowledge the assistance of Colonel William S. Stone of the Office of the Surgeon General and Doctor Austin M. Brues of the Argonne National Laboratory for their efforts in making this work possible. In addition, we are indebted to the Abbott Research Laboratories of North Chicago and to the Ely Lilly Research Laboratories of Indianapolis for their assistance and for the generous supplies of heparin, toluidine blue, and protamine sulfate placed at our disposal.

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ABNORMAL BLEEDING

1. AN EVALUATION OF THE IN VITRO CLOTTING TIME OF WHOLE BLOOD*

by

J. G. Allen, P. V. Moulder, and D. M. Enerson

The value of the whole-blood clotting time determination is questioned by several investigators. Some believe it to be totally useless while others recognize it to be of limited value. The multiplicity of methods and their innumerable modifications support these opinions.

Although methods have been developed using isolated and relatively purified clotting systems with reproducible results, such data are not necessarily applicable to the in vitro clotting power of whole blood. Since the whole blood, and not the purified system, is the physiologic mechanism of normal coagulation, it is necessary to explore existing methods or to develop new approaches in order to evaluate this process.

The status of coagulation was well defined by Joseph Lister⁽¹⁾ in 1858 and is all too applicable today. He wrote:

"Here, then it appears to me, we have a sure, though imperfect glimpse, of the operation of the mysterious but potent forces, peculiar to the tissues of living beings, and capable of reversing the natural order of chemical affinities; forces which I suspect will never be fully comprehended by man in his present state of existence and the study of which should always be approached with humility and reverence."

The nature of the surface that the blood contacts greatly influences its rate of coagulation. John Hunter⁽²⁾ believed that the surface had to be in "harmony with blood" if blood were to remain fluid and that "complete harmony" existed only between blood and the vessel walls. Astley Cooper and Turner Thackrah⁽³⁾ suggested an active role for the blood vessels in preventing coagulation. They observed that blood introduced into the ureter or into a dead vein clotted, whereas blood isolated between two ligatures of the intact vein did not clot for many hours. Bruecke⁽⁴⁾ confirmed these observations and thought that if the influence of the surface of the vessel

*This work was supported by the Medical Department, U. S. Army, the Biological and Medical Research Division of the Argonne National Laboratory, and the Department of Surgery of the University of Chicago.

in preventing intravascular coagulation was due only to its smoothness, then fibrin should not form about spherules of mercury introduced into the heart, since he believed the surface of the endocardium could hardly be smoother than that of mercury. Fibrin did form on the surface of the mercury spherules so he, like Cooper and Thackrah, concluded that the vessel walls, in addition to being smooth, had some property that actively prevented coagulation.

Haycraft⁽⁵⁾ in 1883 demonstrated that blood on a paraffin-coated surface clotted more slowly than on glass. This surface phenomenon has been repeatedly observed and, more recently, other surfaces have been studied, including polythene containers and silicone-coated glassware.⁽⁶⁾ These experiments, while emphasizing the surface phenomena, do not indicate the importance of trauma incident to venepuncture as a factor influencing the in vitro blood clotting time.

This paper presents data that suggest that the normal in vitro whole-blood clotting time is primarily a function of the quantity of thromboplastin produced at venepuncture and of the type of surface the blood contacts. The principle of the method employed is not original.

CS 1-19-51

METHOD

Equipment: All equipment is chemically clean and unscratched.

1. Five serology tubes (1.0 cm x 8.0 cm)
2. One serology tube (1.2 cm x 10.0 cm)
3. One 5-ml glass pipette, graduated to tip
4. One 10-ml glass-tipped syringe coated with thin layer of light mineral oil
5. One 18-gauge needle, 3.8 cm in length

Procedure:

1. Perform venepuncture, using brief stasis, if necessary. If vein is not immediately entered, the needle is discarded and a new attempt is made. There can be no compromise on technique at this point. Allow 8 to 10 ml of blood to flow into syringe without suction. The syringe must be free of bubbles.

2. Blood transfer:

- a. Remove needle from syringe tip
- b. Allow blood to run gently down side of the larger serology tube (1.2 cm x 10.0 cm), except for the last 2 to 3 ml, which are discarded. Little or no pressure is exerted upon syringe plunger.
- c. Fill graduated 5-ml pipette gently with blood aspirated from tube; avoid bubbles
- d. Allow 1 ml to run from pipette down side of each of the five serology tubes (1.0 cm x 8.0 cm)

3. Read clotting time (room temperature $25^{\circ} \pm 3^{\circ}\text{C}$)

- a. Start time recording as soon as the blood flows into view in the syringe. After 15 min., gently tilt the first tube every 3 to 4 min. until clotted. End-point for any tube is the time at which it can remain inverted without flow of blood down the side. After the first tube has clotted, tilt the second tube every two minutes until clotted. Continue this procedure until a tube is found clotted when first examined or until clotting has occurred in the fifth or last tube. By this technique, the clotting time for the normal dog is 20 to 35 min., and for man, 25 to 35 min.

Sources of error:

1. Poor needle puncture technique. A poor venepuncture shortens the clotting time.
2. Air bubbles in the syringe or pipette. Unclean or scratched glassware also causes an undue shortening of the clotting time.
3. Inaccuracies in the volume of pipetted blood and variations in the diameter of the serology tubes. Since the end-point of coagulation by this technique is in part a function of the action of the forces of gravity against the forces of surface tension when the tube is inverted, it is important that the caliber of all the tubes and their contained blood volumes be similar.

EXPERIMENTAL OBSERVATIONS

Clotting time values for normal dogs: Presented in Table 1 is a series of 608 clotting times determined consecutively on 325 normal dogs over a 4 1/2-year period. These data are unselected and include values obtained by personnel in the early phases of their training. The data are arranged by yearly intervals except for the years 1946-47, which are combined. The mean values for these respective periods are 16, 23.9, 25.2, and 28.5 min. The data are also summarized in Figure 1, and indicate improvement in technique though using the same method. At the present time, clotting values of normal dogs (see Part 2 of this report for statistical analysis of these data) of less than 20 min. are considered indicative of technical error and are not accepted unless rechecked. Using this method, a series of daily clotting times of 8 normal dogs is presented in Table 2.

Vessel puncture: Imperfect needle puncture results in accelerated *in vitro* clotting time as indicated in Figure 2. These clotting times were collected from an animal given heparin, the blood samples being drawn simultaneously from separate extremities by two individuals, one skilled in vessel puncture and the other not. A similar observation is presented in Figure 3 in which the *in vivo* tolerance for heparin was determined and in which one imperfect vessel puncture was made. A satisfactory venepuncture is defined as the immediate entrance into either the artery or vein without secondary manipulation and without passing through the vessel which requires partial withdrawal of needle to re-enter lumen. No differences were noted between arterial and venous puncture so long as the vessel puncture was satisfactory.

Effect of temperature: Since it was known that cooling of blood retards coagulation^(7,8) and since the above described procedure was carried out at room temperature, it seemed likely that the clotting times reported herein were in part a function of temperature. Data that indicate this to be true are presented in Table 3. These data were collected from blood drawn by a single venepuncture, drawing 17 ml of blood following a satisfactory vessel puncture. Six ml of blood were placed in each of two serology tubes (1.2 cm x 10.0 cm), the remaining 5 ml of blood being discarded. Using separate 5-ml graduate pipettes, one ml of blood was pipetted into each of five smaller serology tubes (1.0 cm x 8.0 cm). One series was incubated at room temperature (27°C) in air and the other at 37°C in a water bath. In half of the animals the first portion of the syringe sample was read at 37°C and the second portion was read at 27°C. In the remaining half of the animals, this initial portion of the syringe content was pipetted and read at 27°C and the remaining portion of the syringe content was observed at 37°C. Thus any differences that could be attributed to whether the clotting series represented the first or second portions of blood to enter the syringe were

Table 1

Clotting times for 325 normal dogs

	Years			
	1946-1947	1948	1949	1950 (2 mo.)
	Total number of clotting times			
	100	78	377	53
Clotting times				
Less than 10 min.	16	3	2	
11 "		1	2	
12 "	10	3	4	1
13 "	1		1	
14 "	8		2	
15 "	2	2	6	
16 "	6	3	2	
17 "	5	1	2	1
18 "	10	1	3	
19 "	8	1	7	
20 "	8	4	24	1
21 "	4	5	10	2
22 "	8	2	13	1
23 "	1	5	12	2
24 "	3	7	19	2
25 "	3	5	29	2
26 "	3	6	20	2
27 "		3	22	5
28 "	2	3	29	3
29 "		3	18	4
30 "		6	34	12
31 "		4	12	4
32 "	1	2	13	1
33 "	1	1	18	1
34 "		3	13	1
35 "		2	16	
36 "		1	11	3
37 "			7	
38 "			5	2
39 "		1	1	1
40 "			10	
Average time (min.)	16	23.9	25.2	28.5

NORMAL WHOLE BLOOD CLOTTING TIMES

Figure 1. Whole-blood clotting time values for 325 normal dogs.

Table 2

Series of daily clotting times for eight normal dogs

1949

Dog no.	Days												
	1	2	3	4	5	6	7	8	9	10	11	12	13
959	22	27	31	37	25	32	30	29	23	25	22	19	35
132	15	35	35	25	30	30	36	36	35	15	35	36	38
48	25	30	30	31	30	25	30	27	38	33	30	40	
763	27	28	27	33	31	30	28	28	30	29	27	28	
960	27	24	27	32	31	34	31	35	28	32	28		
232	36	32	29	29	35	29	28						
832	31	22	35	35	35	35							
258	34	25	32	25	27	24							

equalized. In general, the samples read at 37°C were shorter than those incubated at room temperature.

The effect of pipetting on the platelet count: Since platelets are known to adhere to glass surfaces, it was anticipated that the platelet concentration of the pipetted samples would decrease serially in the five tubes. However, no constant difference was observed either in the normal dog or in the dog with pancytopenia induced by X irradiation (Table 4). These findings were further substantiated in that no clotting time differences were noted when the reading procedure was reversed, beginning at the fifth tube instead of the first.

Silicone-coated equipment: The procedures described above were also performed at room temperature using silicone-coated equipment. Normal clotting time values greater than one hour were obtained. In dogs with abnormal clotting times, the blood frequently failed to clot in 12 hours even though clotting times using uncoated glass were in the range of 75 to 90 min.

The clotting times obtained when all or portions of the apparatus used were coated with silicone are presented in Table 5.

Figure 2. Comparison of effect of good and bad venepuncture technique upon whole-blood clotting time.

Figure 3. Result of poor venepuncture technique upon the whole-blood clotting time.

Table 3

Whole-blood clotting times at 27°C and 37°C

Dog no.	Temperatures	
	27°C	37°C
	Time (min.)	
160	36	36
161	47	35
601	44	25
665	45	25
999	35	30
726	41	30
555	30	25
437	34	25
839	43	25
861	40	20
840	38	25
860	26	26
845	27	27
854	45	19
846	37	27
556	26	19
557	20	21
343	27	14
344	25	12
691	23	19

Table 4

Platelet counts for normal and pancytopenic dogs in a whole-blood clotting time study

Dog no.	Tube No.					
	Normal Platelet count	1	2	3	4	5
417	320,000	330,000	310,000	250,000	190,000	180,000
441	100,000	110,000	90,000	80,000	100,000	100,000
406	12,000	14,000	10,000	12,000	10,000	12,000
645	440,000	450,000	390,000	380,000	410,000	420,000
P ₁	320,000	320,000	342,000	300,000	326,000	322,000
690	310,000	340,000	300,000	280,000	300,000	294,000
P ₂	200,000	220,000	200,000	198,000	-	196,000
413	88,000	94,000	88,000	96,000	90,000	92,000

Table 5

Changes in normal whole-blood clotting time at room temperature produced by coating various portions

Needle	Syringe	Pipette	Tubes	Dog A	Dog B
Coating				Whole-blood clotting time in min.	
-	-	-	-	34	30
oil	-	-	-	37	36
silicone	-	-	-	28	32
-	silicone	-	-	33	31
-	-	silicone	-	25	29
-	-	-	silicone	38	53
silicone	silicone	silicone	-	67	62
silicone	silicone	silicone	silicone	132	64

DISCUSSION

There are no errors in the technique of performing clotting time measurements on whole blood that lengthen the clotting time unless inhibitors are introduced by unclean equipment. All other errors that exert an influence invariably result in an accelerated clotting time. Outlined under METHOD are some of the more frequently encountered errors, any one of which is capable of accelerating in vitro whole-blood coagulation. In our experience the most important errors are introduced during the withdrawal of blood, especially when the vessel puncture is not prompt or not made with minimal trauma. Errors at this point result from added thromboplastin produced by trauma and cannot be overcome by coating the equipment with silicone, since tissue thromboplastin entering the syringe, etc., in this manner is active regardless of silicone coating. The importance of technical skill in the vessel puncture is obvious.

The data provided by the whole-blood clotting time often cannot be obtained by any other procedure. For example, there are certain theoretical as well as practical considerations that distinguish between the whole-blood clotting time and the one-stage prothrombin determination. While these differences are important to each procedure, they are at the same time mutually exclusive. For example, the prothrombin test is subject to less error when thromboplastic activity is maximum and coagulation proceeds with greatest rapidity. While providing uniform results, it is objectionable in that any thromboplastin deficiency or inhibitor will be masked in the process. This has been demonstrated in our laboratory in three patients with agnogenic hemorrhagic disease⁽⁹⁾ and more recently by Tocantins⁽¹⁰⁾ in patients with hemophilia.

Since the amount of thromboplastic activity necessary to accelerate the in vitro clotting time of whole-blood is small, it is necessary to reduce tissue contaminants to a minimum if this type of procedure is to register the presence of inhibitors of the first phase of coagulation. It is for this reason the clotting time of whole blood of the therapeutically heparinized dogs and patients is prolonged while the prothrombin activity remains relatively normal. As shown in Figures 2 and 3, the anticoagulant effect of an inhibitor as potent as commercial heparin can be partially or even completely overcome by excessive trauma at venepuncture.

Minimizing the acceleration of clotting times may also be important in the demonstration of inhibitors in the second phase of coagulation. Antithrombin, for example, requires a 15-min. incubation period at 37°C with thrombin before its complete effect is apparent⁽¹¹⁾, and even longer at room temperature. Hence, accelerated clotting times of 5 to 15 min. induce coagulation before antithrombin can exert its influence.

Unfortunately, changes in the clotting time do not necessarily forecast hemorrhage or thrombosis. In fact, none of the in vitro clotting procedures offers any explanation as to why the individual should spontaneously bleed when an abnormality in the clotting mechanism is present. These questions can only be answered by further research, and until such time we must be satisfied with the high incidence of spontaneous hemorrhage that accompanies the finding of specific abnormalities.

CONCLUSION

Although the in vitro clotting mechanism is best studied with both whole blood as well as with purified systems, the whole-blood clotting time is of little value unless more care is directed to details in technique. When sufficient technical skill is applied, especially with reference to the venepuncture, reproducible clotting times of 25 to 35 min. range can be obtained at room temperature with uncoated glassware in dogs and man. Under these conditions the whole-blood clotting time is a useful tool in experimental and clinical research.

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2. CLOTTING TIME CHANGES AFTER TOTAL-BODY X IRRADIATION

A STATISTICAL ANALYSIS*

by

J. G. Allen, L. J. Savage, P. V. Moulder, and D. M. Enerson

In our experiments 450 r total-body X irradiation produced an increase in the clotting time of whole blood of dogs, and while others have observed this same phenomenon^(1,2) some have not.^(3,4)

This report is an analysis of the clotting times of 79 irradiated dogs studied consecutively from July 1, 1948 to February 1, 1950. The clotting time measurements were made by the same personnel, and the data obtained are illustrated in Figure 4. The platelet counts made daily on this same series of animals are also presented.

A statistical analysis was made by one of us (L.J.S.) of the series of dogs studied during 1949 to ascertain the significance of the observed changes in the clotting times. This year was chosen because it represents a reasonably large homogeneous body of data under nearly constant laboratory conditions. For this group, the mean control clotting time is 28.5 min. which with 99 per cent confidence may be said to lie within 1.8 min. of the stated figure.

Information on the variabilities of repeated control determinations of the same dog may be summarized as follows:

	<u>Estimate</u>	<u>99% confidence interval</u>
Variance	31.50 min. ²	28.54 to 34.53
Standard deviation	5.61 min.	5.34 to 5.88

The variability of the clotting time attributable to differences between dogs during the control period was actually less than the variability of multiple determinations on the same animal:

	<u>Estimate</u>	<u>99% confidence interval</u>
Variance	4.67 min. ²	0.00 to 6.62
Standard deviation	2.16 min.	0.00 to 2.57

*This work was supported by the Medical Department, U. S. Army, the Biological and Medical Research Division of the Argonne National Laboratory, and the Department of Surgery of the University of Chicago.

Figure 4. Average whole-blood clotting time and platelet count for 79 dogs after single total-body exposure to 450 r X ray.

Returning to the estimate of 5.61 min. for the standard deviation of measurements on one dog under control conditions, it can roughly be concluded that if N dogs ($N \geq 5$) are measured once each in the control and then in some treated state, and if the treatment induces a change d in the mean clotting time that there are about 9 chances in 10 of detecting the change (and calling it significant at the .05 level), provided d is even as big as $d_0 = 3.28 \times \sqrt{\frac{2}{N}} \times 5.61$, i.e.,

$$d_0 = 25.95 / \sqrt{N} \text{ min.}$$

for example:

N	9	16	25	49	100
d_0	8.7	6.5	5.2	3.7	2.6 min.

Estimates of mean and standard deviation from dog to dog were computed for several postirradiation days and are presented in Table 6. Under the conditions of the experiment, the standard deviation tends to increase very rapidly with the mean. The relationship between the mean and standard deviation is roughly given by the following equation estimated graphically from Table 6:

$$\begin{aligned} \text{S.D.} &= 0.87 m - 19.5 \\ &= 0.87 (m - 22.3) \end{aligned}$$

Table 6, and equivalently the equation above, show that considerably larger variations in the clotting time may be expected when the mean value is considerably elevated. This has the practical consequence that a given absolute, and to a lesser extent relative, difference between two similar treatments will become harder to detect as the mean clotting times induced by the treatments increase.

This larger variation when the mean value is elevated is in a large part the result of minor changes in thromboplastic substances produced by imperfect venepuncture or handling of the blood as indicated in the preceding paper, and those factors which exert an effect on the clotting time have a greater influence on the clotting time when it is prolonged than when it is normal (Part 3).

Figure 4 makes it clear, in our opinion, that clotting times tend to be elevated after irradiation, except possibly on the first few days and also when too few dogs remain alive in the later days to provide data. Objective support of this contention is provided in Table 7, which shows for each of three days how many dogs had higher and how many lower clotting times than in their control periods. These numbers depart from the 50:50 ratio

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Table 6

Mean clotting times and standard deviations in postirradiation
clotting times of dogs

1949

Day	1	2	3	4	5	6	7	8	9	10	11
Number of dogs observed	43	30	39	32	40	38	41	38	24	28	20
Mean clotting time	30.65	30.53	31.74	35.12	34.50	42.08	46.71	50.34	50.29	43.57	54.90
Standard deviation	7.12	9.25	6.35	8.44	7.82	19.3	18.00	23.98	21.10	17.75	22.60

Table 7

Three-day comparison of clotting times with control periods

Day	Higher	Lower	P
1	40	20	.02
8	58	9	10^{-8}
12	30	5	10^{-4}

which would be expected if irradiation were without effect on clotting time. The probabilities of departure as great as that observed occurring on the basis of chance alone is also shown.

It is seen that there is some evidence that the clotting time is increased on the first day after exposure. The validity of the clotting time increase on the eighth day is indisputable and on the twelfth day is still excellent. Though further evidence is not called for, it may be of interest to note that while no clotting time in the control period was as high as 45 min., 74 of the 79 dogs attained at least this value some time after irradiation.

To summarize, ^{2/ indicated} it is beyond doubt that the evidence pointing to increased clotting time after irradiation is not attributable to chance alone. ^{2/ IE ND}

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3. INDEPENDENCE OF PROTAMINE TITRATION AND PLATELET LEVEL IN CERTAIN HEMORRHAGIC DISEASES*

by

P. V. Moulder and J. G. Allen

INTRODUCTION

Following the addition of a standard quantity of heparin to normal blood, the amount of protamine sulfate required to permit this blood to clot within one hour is remarkably constant providing the reagents are of constant potency.⁽¹⁾ Although species differences do exist, the protamine required for members of the same species varies to only a slight degree.

This protamine requirement as determined by the protamine titration⁽¹⁾ may be increased in certain pathologic states associated with hemorrhage. Increased protamine requirements in whole blood of patients have been observed in association with acute leukemia, chronic leukemia, some cases of idiopathic thrombocytopenia, secondary thrombocytopenia, aplasia of the bone marrow, and in some cases where no other abnormalities were evident. Increases have also been observed in the toxic states associated with total-body X irradiation, nitrogen mustards, and aminopterin therapy. Many but not all of these conditions are associated with thrombocytopenia. The hemorrhagic complications of these disorders are often temporarily improved and the protamine titration returned to normal by the administration of toluidine blue, even though the platelet count is unchanged. An increased protamine titration has also been found in bleeding patients who have normal platelet counts and normal prothrombin activity.⁽²⁾ These patients likewise respond to toluidine blue and protamine therapy, and the protamine titrations return to or toward normal.

These and other observations suggest that the increased protamine titration may be due to a disturbance in the clotting mechanism which in some ways is similar to that produced by the intravenous injection of commercial heparin. However, certain distinct differences between this heparinoid condition and that produced by the intravenous injection of commercial beef heparin have been demonstrated in both man and the dog.⁽³⁾ The principal difference appears to be in anticoagulant potency for the blood of patients, and the blood of dogs with these disorders rarely inhibited

*This work was supported by the Medical Department, U. S. Army, the Biological and Medical Research Division of the Argonne National Laboratory, and the Department of Surgery of the University of Chicago.

or delayed coagulation of normal blood. Furthermore, the protamine titration was increased in many of our patients and experimental animals to such an extent that, had the increased protamine titration resulted from heparinemia identical with that produced by an injection of commercial beef heparin (Abbott), the blood would invariably have been incoagulable.

Recently, Conley, Hartmen, and Lalley⁽⁴⁾ have observed that normal human plasma could be made more sensitive to the effects of added heparin by removing most of the platelets by centrifugation. They concluded that these plasma data were applicable to the protamine titration and that these changes "can be explained by the variation in the platelet concentration alone."

Observations of the type reported by the Hopkins group in 1948 were investigated and reported by us in 1947⁽⁵⁾ in the course of development of the protamine titration. At that time we stated "Preliminary evidence is presented which suggests that the blood of the thrombocytopenic patient tolerates heparin less well than does normal blood." We found that prothrombin deficient and thrombocytopenic bloods were more sensitive to the effects of added heparin, both in vivo and in vitro. In this connection the earlier observations of Howell⁽⁶⁾ in 1918 are of interest. He observed that the effects on the amount of heparin just sufficient to prevent clotting could be overcome by cephalin obtained from brain tissue, recognizing this as a substance also found in platelets.

Conley et al. were in error when they assumed that their measurements of heparin sensitivity in thrombocytopenic plasma could be extended to include the protamine titration, which they did not report as having performed. That this assumption is not valid is shown by the fact that heparin sensitivity and the protamine titration measure two different phenomena. These phenomena frequently, but not invariably, co-exist but appear to be independent. It is apparent that Conley's data confirm our previous report and are in no way contradictory to our observations concerning heparin sensitivity in thrombocytopenia. This has also been recently confirmed by Carr and Fowler.⁽⁷⁾ The protamine titration may be normal in severe thrombocytopenia and it also may be markedly increased (abnormal) in patients with normal platelet counts, normal prothrombin times, and even normal clotting times, as illustrated by the following observations.

To produce an increase in the protamine titration of the smallest increment used, namely 0.020 mg of protamine, a minimum of 0.018 mg of beef heparin must be added to each ml of blood tested. This was 2 to 5 times the amount of heparin required to increase the clotting time of whole blood or plasma in vitro, and was 25- to 50-fold greater than that required to increase the clotting time of "platelet-free" plasma,⁽⁴⁾ yet the conditions in which increases in the titration occur are associated with only a slight

to moderate increase in the whole-blood clotting time. These comparisons, however, are based on data obtained from normal human blood and plasma and do not necessarily apply to bleeding disorders. Moreover, it has been observed that animals treated with aminopterin develop an increased protamine titration without thrombocytopenia or an increased in vitro heparin sensitivity.⁽⁸⁾

The protamine titration, however, is affected by several factors, most of which influence fibrin formation. Among these factors are: heparin or heparin-like substances, prothrombin deficiency, impairment of the conversion of prothrombin to thrombin, hemophilia, and the absence of fibrinogen. It is possible to set up a very sensitive protamine titration over a narrow range of protamine concentrations that can be altered by changes in the platelet count, but such a titration would lose its clinical value.

A series of 21 bleeding patients representing a variety of malignant and benign disorders with platelet counts ranging from 180,000 to 324,000 per cu mm with increased protamine titrations is presented in Table 8.

Table 9 shows 16 patients with similar bleeding with thrombocytopenia (20,000 to 108,000 per cu mm) and normal protamine titrations.

Table 10 presents serial studies on three patients suffering from Hodgkin's disease receiving nitrogen mustard therapy showing thrombocytopenia of varying degree and consistently normal protamine titrations.

A group of bleeding patients with abnormal protamine titrations receiving toluidine blue therapy is presented in Table 11. The platelet levels remain low or actually become lower during the period of study while the protamine titration returned to normal in eight patients and toward normal in the remaining six.

Table 12 shows the presence of severe thrombocytopenia with normal protamine titrations in three bleeding patients with idiopathic thrombocytopenia. Four other hemorrhaging patients listed in this table had an increased protamine titration that returned to normal after toluidine blue therapy, although the platelet count was unchanged. Three more patients with normal protamine titrations, severe thrombocytopenia, and bleeding, who did not receive toluidine blue are also listed in this disease category. The detailed studies on these and other patients have been presented elsewhere.⁽²⁾

Table 8

Patients with increased protamine titrations and normal platelet counts

Patient	Diagnosis	Platelets	Protamine titration*
451925	Lymphatic leukemia	270,000	0.160
225-b	Myelogenous leukemia	240,000	0.180
436363	Myelogenous leukemia	270,000	0.180
235-a	Unknown	260,000	0.180
453730	Stem cell leukemia	200,000	0.160
385239	Post-partum hemorrhage	244,000	0.200
122-a	Bleeding tendency	212,000	0.200
166-a	Beryllium toxicity	218,000	0.160
310314	Lymphatic leukemia	192,000	0.180
357543	Hodgkin's disease	202,000	0.200
44-b	Post-partum hemorrhage	180,000	0.160
12-b	Post-partum hemorrhage	260,000	0.400
219-a	Unknown	240,000	0.160
145-a	Menorrhagia	324,000	0.200
40-b	Menorrhagia	184,000	0.160
235-a	Menorrhagia	320,000	0.160
6-b	Menorrhagia	210,000	0.160
226-a	Menorrhagia	290,000	0.180
22-b	Menorrhagia	260,000	0.160
22-b-b	Menorrhagia	230,000	0.160
9-b	Menorrhagia	210,000	0.160

* Following the addition of a standard concentration of heparin to normal blood a remarkably constant amount of protamine is required to restore clotting. (Laboratory normal is 0.140 mg protamine sulfate (Lilly) per ml blood containing 0.091 mg heparin (Abbott).)

Table 9

Patients with normal protamine titrations and low platelet counts

Patient	Diagnosis	Platelets	Protamine titration*
421375	Thrombocytopenia	50,000	0.140
304793	Hodgkin's disease Nitrogen mustard Rx	54,000	0.140
370828	Hodgkin's disease Nitrogen mustard Rx	34,000	0.140
54159	Monocytic leukemia	91,000	0.140
424699	Lymphatic leukemia	64,000	0.140
417601	Lymphatic leukemia	51,000	0.140
424944	Secondary thrombo- cytopenia	73,000	0.140
429888	Lymphatic leukemia	85,000	0.140
391785	Hodgkin's disease Nitrogen mustard Rx	20,000	0.140
15517	Toxic depression of bone marrow	90,000	0.140
D-a22	Allergy?	108,000	0.140
B-119a	Post-irradiation	157,000	0.140
W-113a	Hodgkin's disease Nitrogen mustard Rx	58,000	0.140
F-b36	Easy bruising	108,000	0.140
B.R.I.	Lymphoma?	67,000	0.140
S.C.H. (202a)	Lymphatic leukemia	90,000	0.140

*Following the addition of a standard concentration of heparin to normal blood a remarkably constant amount of protamine is required to restore clotting. (Laboratory normal is 0.140 mg protamine sulfate (Lilly) per ml blood containing 0.091 mg heparin (Abbott).)

Table 10

Representative patients with numerous studies and normal protamine titrations and low platelet counts

Patient	Diagnosis	Platelets	Protamine titration*	Date
304793	Hodgkin's disease Nitrogen mustard Rx	54,000	0.140	1-20-48
		71,000	0.140	1-24-48
		107,000	0.140	1-26-48
		87,000	0.140	1-27-48
		68,000	0.140	1-28-48
		94,000	0.140	2- 4-48
		77,000	0.140	2- 5-48
		76,000	0.140	2-11-48
		73,000	0.140	2-23-48
370828	Hodgkin's disease Nitrogen mustard Rx	47,000	0.140	1-20-48
		34,000	0.140	1-24-48
		56,000	0.140	1-26-48
		69,000	0.140	1-27-48
		73,000	0.140	1-28-48
		65,000	0.140	1-29-48
		98,000	0.140	1-30-48
357210	Hodgkin's disease Nitrogen mustard Rx	114,000	0.140	3- 3-48
		99,000	0.140	3- 5-48
		67,000	0.140	3- 9-48
		69,000	0.140	3-10-48
		50,000	0.140	3-12-48
		97,000	0.140	3-15-48
		67,000	0.140	3-17-48

*Following the addition of a standard concentration of heparin to normal blood a remarkably constant amount of protamine is required to restore clotting. (Laboratory normal is 0.140 mg protamine sulfate (Lilly) per ml blood containing 0.091 mg heparin (Abbott).)

Table 11

Patients with increased protamine titrations and low platelet counts before and after receiving toluidine blue

Patient	Diagnosis	Platelets		Protamine titration*	
		Before	After	Before	After
		Toluidine blue		Toluidine blue	
436363	Acute leukemia Aminopterine Rx	140,000	24,000	0.180	0.160
453730	Acute leukemia	190,000	17,000	0.180	0.140
K-a234	Acute leukemia	20,000	80,000	0.180	0.160
413730	Acute leukemia	52,000	24,000	0.180	0.160
M-a186	Chronic leukemia Spray irradiation	10,000	16,500	0.180	0.160
341183	Hodgkin's disease Nitrogen mustard Rx	124,000	37,000	0.200	0.160
395060	Hodgkin's disease Nitrogen mustard Rx	83,000	85,000	0.200	0.140
409460	Hodgkin's disease Nitrogen mustard Rx	64,000	37,000	0.200	0.140
435361	Hodgkin's disease Nitrogen mustard Rx	81,000	75,000	0.160	0.140
453179	Hodgkin's disease Nitrogen mustard Rx	19,000	70,000	0.160	0.140
T-a229	Toxic pancytopenia	7,000	16,000	0.180	0.140
15517	Toxic depression of bone marrow	85,000	15,000	0.180	0.140
H-b32	Menorrhagia	66,000	72,000	0.160	0.140
423054	Cancer, breast P ³² therapy	100,000	93,000	0.180	0.160

*Following the addition of a standard concentration of heparin to normal blood a remarkably constant amount of protamine is required to restore clotting. (Laboratory normal is 0.140 mg protamine sulfate (Lilly) per ml blood containing 0.091 mg heparin (Abbott).)

Table 12

Patients with idiopathic thrombocytopenia purpura
(Werlhof's Disease)

Patient	Platelets		Protamine titration*	
	Before Toluidine blue	After Toluidine blue	Before Toluidine blue	After Toluidine blue
432419	37,000	40,000	0.140	0.140
H-a103	2,000	3,000	0.140	0.140
424944	60,000	83,000	0.140	0.140
416318	52,000	68,000	0.200	0.140
235457	60,000	65,000	0.160	0.140
Patient	Platelets		Protamine titration	
E-a52	99,000		0.140	
B-a12	96,000		0.140	
Y-a106	66,000		0.140	

* Following the addition of a standard concentration of heparin to normal blood a remarkably constant amount of protamine is required to restore clotting. (Laboratory normal is 0.140 mg protamine sulfate (Lilly) per ml blood containing 0.091 mg heparin (Abbott).)

DISCUSSION

These data indicate that the protamine titration may be increased in the presence of normal platelet counts as well as in severe thrombocytopenia. Moreover, the protamine titration can be returned to normal by the administration of adequate amounts of toluidine blue without materially altering the platelet count. In addition, thrombocytopenia may occur without increasing the protamine titration.

The protamine titration is probably increased by factors that directly or indirectly delay the formation of fibrin. Abnormal values in the protamine titration cannot be interpreted unless all known clotting factors can be evaluated. The titration can be increased when large quantities of heparin or heparin-like substances are present, but it does not necessarily follow that the factor(s) responsible for the increased protamine titration in these patients was actually heparin or even heparin-like. Some endogenous compounds, not of the heparin family, were found to be mild anticoagulants and were inhibited by both toluidine blue and protamine sulfate.⁽³⁾ The exact nature of the factor(s) responsible for the defect(s) observed in these patients remains unknown.

SUMMARY

1. The platelet concentration influences the sensitivity of blood and plasma to the effect of added heparin. The anticoagulant potency of heparin is also enhanced by prothrombin deficiency. The potentiated effect of heparin in thrombocytopenic blood may be due to reduced thromboplastin (cephalin) activity.
2. The protamine titration may be increased when the platelet count is normal and it may be normal in the presence of thrombocytopenia. It is influenced by heparin, heparinoid substances, prothrombin deficiency, hemophilia, and possibly other factors.
3. Increased protamine titrations may occur in bleeding patients who have no other apparent clotting defects, including the whole-blood clotting time. Many of these patients cease bleeding when given intravenous injections of toluidine blue, following which the protamine titration may be returned to or toward normal regardless of the platelet level.

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4. SOME OBSERVATIONS ON THE RELATIONSHIP OF HEPARIN TO PROTAMINE SULFATE AND THE BLOOD PLATELETS*

by

J. G. Allen, P. V. Moulder, M. H. Sanderson, and W. M. Egner

INTRODUCTION

In all of the experiments to be detailed in this and subsequent sections, the data to be presented were obtained from studies of the effect of a single total-body exposure of 450 r.⁽¹⁾ This experimental approach has been pursued because the bleeding, irradiated dog and patients with certain hemorrhagic states have certain defects in common.⁽¹⁻³⁾ The methods employed upon which these comparisons were made include: 1) the whole-blood clotting time, 2) heparin sensitivity in vivo and in vitro, and 3) the protamine titration.⁽⁴⁾ In conjunction with these methods, certain other procedures have been devised and form the basis for this and subsequent reports. Since some of these are not adaptable for study in the bleeding patients, this report will be confined to experimental observations carried out in the dog. While these data cannot yet be interpreted in terms of human observations, it is hoped that they eventually may clarify some aspects of abnormal bleeding in human subjects.

Whole-blood clotting time procedure, see Part 1.

Heparin sensitivity.

In vitro heparin sensitivity herein described is determined on whole blood. The same precautions for the drawing and handling of blood described for the clotting time procedure were employed in this determination.

Equipment:

- Eleven serology tubes (1.0 cm x 8.0 cm)
- One 15-ml centrifuge tube
- One 10-ml graduated glass pipette, marked to contain 11 ml
- One 20-ml, oil-coated glass syringe
- One 18-gauge needle, 3.8 cm in length
- One 0.1-ml micropipette

*This work was supported by the Medical Department, U. S. Army, the Biological and Medical Research Division of the Argonne National Laboratory, and the Department of Surgery of the University of Chicago.

Procedure:

Draw venous blood into an oiled syringe by a satisfactory femoral needle puncture. Gently transfer the blood to the centrifuge tube, allowing the blood to flow gently down the side of the tube. Aspirate 11 ml of this blood into the graduated pipette, carefully avoiding air bubbles. Allow 1 ml of blood to flow from the pipette gently down the side of each of the 11 serology tubes. Tube numbers 2 through 10 contain previously added heparin in volumes not exceeding 0.1 ml. Tubes 1 and 11 contain nothing but blood and serve as controls. The quantity of heparin contained in this 0.1-ml volume is varied as the occasion demands (see EXPERIMENTAL). Shake rack of tubes in the horizontal plane for 20 oscillations of the wrist to achieve reasonable mixing of the blood and heparin.

The clotting end-point is determined by tilting the entire rack of tubes at 3-min. intervals until fluidity is no longer readily apparent for any one tube. Each tube is then removed as the blood it contains gels, and the final clotting end-point is determined for each tube by the original Lee-White procedure⁽⁵⁾ as distinguished from clotting time described in Part I. Sources of error are similar in nature to those encountered in the whole-blood clotting time outlined above. In addition, heparin of proven and known potency must be used and pipetting errors avoided.

In vivo heparin sensitivity

This procedure will be described under EXPERIMENTAL.

Protamine titration

This procedure is employed as described elsewhere.⁽⁴⁾

Erythrocyte, leucocyte, and platelet counts are made daily on all normal and irradiated animals under observation.

Prothrombin determination.⁽⁶⁾

EXPERIMENTAL

Heparin sensitivity: Heparin is best known for its antithrombic activity, but it is also an antagonist of thromboplastin. Howell⁽⁷⁾ observed that when blood is prevented from clotting by the addition of an excess of heparin, the further addition of cephalin may not be adequate to induce clotting. However, when the amount of heparin used is not in excess but is sufficient simply to delay the process of coagulation, then cephalin antagonizes its action and brings on prompt clotting.

Since platelets are rich in cephalin, it seemed logical that the blood of thrombocytopenic patients should tolerate heparin less well than normal blood. This we investigated and demonstrated in patients in 1947,^(8,9) and our results have been confirmed by Conley *et al.*⁽¹⁰⁾ and Rosenthal.⁽¹¹⁾

A more detailed resume of our findings relating to factors influencing heparin sensitivity are presented below.

In vitro heparin sensitivity

Normal dog. When constant volumes of blood are placed in serology tubes containing known increments of heparin,* the least amount of heparin that will prolong the clotting time ranges between 2 and 3 μ g per ml of blood (Table 13). With technical errors in handling blood, this amount may be considerably increased (6 to 10 μ g).

Prothrombin deficiency. The sensitivity of prothrombin deficient blood (dicumarol-induced) to added heparin is increased over that of normal blood. Such increases are not pronounced until prothrombin deficiency is marked (Figure 5). In general, no increase in heparin sensitivity is noted until the prothrombic activity is reduced to less than 40 per cent of normal. It is at this level of prothrombin deficiency that the first increases in the whole-blood clotting time can be noted.⁽⁹⁾

Thrombocytopenia induced by irradiation. This state is complex and factors other than thrombocytopenia are also present. The in vitro heparin sensitivity of blood in the postirradiated state, however, is greatly increased. In many instances as little as 1/10 to 1/30 the minimal amount of heparin required to increase the in vitro whole-blood clotting time of blood in the pre-irradiated state, is effective later on (Figures 6 and 7). From the data in Figures 5, 6, and 7, it is clearly evident that both prothrombin deficiency and thrombocytopenia can create striking

*100 units per mg.

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Table 13

In vitro heparin sensitivity of whole blood of normal dogs

Dog no.	Control	Micrograms of heparin / ml blood									Control
		1	2	3	4	5	6	7	8	9	
571	8*	-	14	43	67	80	6 hr	6 hr	--**	--	9
575	15	-	48	74	6 hr	6 hr	6 hr	--	--	--	15
623	15	25	37	63	84	94	113	218	15 hr	--	23
625	15	44	49	80	143	246	6 hr	6 hr	6 hr	--	16
460	15	15	51	135	135	375	375	375	375	18 hr	15
699	16	16	39	39	49	49	105	140	445	445	12
717	12	22	26	52	200	420	420	24 hr	24 hr	24 hr	17

*Time in min. from time of shaking rack, to insure mixing, 15 times in rotating motion.

**Indicates time was in excess of 24 hr.

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Figure 5. Effect of hypothyrombinemia on the sensitivity of whole blood to heparin.

Figure 6. Dog 498.

Increased sensitivity of the whole-blood clotting time to increasing increments of added heparin in the postirradiation period. Heparin concentrations are on a semi-logarithmic scale, indicating the profound sensitivity of these bloods.

Figure 7. Dog 422.

Increased sensitivity of the whole-blood clotting time to increasing increments of added heparin in the postirradiation period. Heparin concentrations are on a semi-logarithmic scale, indicating the profound sensitivity of these bloods.

increases in sensitivity to added heparin. Because of the different mechanisms of actions of these two deficiencies, there is no common ground upon which their respective roles in heparin sensitivity can be compared. The impression that we have gained, however, is that small changes in the thrombocyte count are more likely to influence heparin sensitivity than are small variations in prothrombic activity. Although in severe prothrombin deficiency, minor variations in prothrombin activity result in major changes in heparin sensitivity.

Aminopter-in-treated dogs. In another report,⁽¹²⁾ it was noted that aminopter-in (4-amino-ptyryl glutamic acid) administered to normal dogs resulted in moderate bleeding with an increased protamine titration but without thrombocytopenia. Because an increased protamine titration and thrombocytopenia often occur simultaneously in the irradiated dog, the aminopter-in-treated dog was employed so that heparin sensitivity could be studied under conditions in which there was an increased protamine titration without thrombocytopenia. Representative data (Figure 8) show that no increase in heparin sensitivity developed during aminopter-in administration even though definite increases in the protamine titration occurred. This would seem to support our previous observations that the changes in the protamine titration and the heparin sensitivity are largely independent of one another.⁽⁹⁾

In vivo heparin sensitivity.

Normal dog. When standard quantities of liquid commercial bovine heparin (Abbott) are administered intravenously to normal dogs on a mg per kg basis, the clotting time response is more constant than is generally appreciated, providing the technical details of measuring the clotting time are mastered. Data representative of 25 normal dogs given 0.5 mg of heparin per kg of body weight are presented in Figure 9. The variations in response are greatest at the height of the curve (the first 15-min. sample). This phenomenon has also been observed in vitro where the effect of increasing increments of heparin on the clotting times is also geometric. This being so, the lower portions of the curve are less sensitive to added heparin and, therefore, the period of time between the heparin injection and the return of the clotting time to normal is similar for different dogs under comparable conditions.

Clotting time changes following the injection of different quantities of heparin on a per kg basis are illustrated in Figure 10.

Figure 8. Dog 959.

Daily in vitro sensitivity of whole-blood clotting time to added heparin.

Figure 9. In vivo heparin sensitivity range of response of whole-blood clotting time of normal dogs to 0.5 mg per kg body weight of heparin (Abbott) administered intravenously.

Figure 10. Effect of intravenous heparin on whole-blood clotting time in normal dogs.

Not only is the immediate increase of the clotting time greater or less in accordance with the size of the dose, but the duration of detectable effect also is determined largely by the size of the dose.

A distressing feature in this type of experiment is the effect a poor needle puncture has upon the clotting time, as previously described. Such errors can completely mask minor or even major increases in the clotting time and may account for some of the difficulties that Holden *et al.*⁽¹³⁾ and Rosenthal and Benedek⁽¹⁴⁾ encountered in their unsuccessful attempts to find the clotting time prolonged after irradiation.

Prothrombin deficiency. When the normal dog is given dicumarol, the *in vivo* sensitivity to heparin increases as the prothrombin deficiency becomes pronounced. While the sensitivity at 5 to 10 per cent of prothrombin activity is as great as that observed in thrombocytopenia, there is little increase in heparin sensitivity when the prothrombin level is above 50 per cent of normal. It seems likely that when both prothrombin deficiency and thrombocytopenia are present, the animal's sensitivity to heparin may be greater than when only one of these deficiencies exists.

Irradiation thrombocytopenia. We are not aware of any means for inducing experimental thrombocytopenia in the dog which does that and nothing more. We have been unable to accurately reproduce in the dog the marrow and peripheral blood pictures of human idiopathic thrombocytopenia (Wärthof's syndrome). The observations reported here were obtained on irradiated dogs and are more complex than is desirable for the purpose of the experiments. Many of these dogs showed an increase in heparin sensitivity, increases in the protamine titration, increases in the plasma antithrombin activity, and changes in the vessel walls of capillaries and small vessels.

In one series of observations, normal and irradiated dogs were given daily intravenous doses of 0.5 mg of heparin per kg of body weight. The clotting times were determined before and 90 min. after the daily heparin injections. The results obtained are presented in Figure 11. The presence of an increase in heparin sensitivity in the irradiated animal is indicated by the prolongation of the clotting time, measured 90 min. after heparin injection. The decline in platelet number mirrored the increase in heparin sensitivity. The prothrombin times in neither the normal nor in the irradiated animals were appreciably changed.

Figure 11. Response of the whole-blood clotting time in normal and irradiated dogs 90 min. after the intravenous daily injection of 0.5 mg per kg heparin.

The animal's sensitivity to heparin was 10 to 30 times that of the normal animal (Figures 10 and 12). Data obtained from a dog given 0.1 mg heparin per kg daily before and after irradiation are shown in Figure 13. This amount produced no clotting change in the pre-irradiation period but from the 4th day after exposure; the daily injections produced progressively increasing effects.

Aminopterin-treated dog. In order that the role of increased protamine titration without thrombocytopenia might be assessed in vivo with reference to heparin sensitivity, protamine titrations and heparin sensitivity studies were made on dogs in the course of aminopterin administration. The results disclosed an increase in the titration but showed no change in heparin sensitivity. These data correspond to the results also obtained with in vitro methods.

Protamine titration:

Effect of in vitro heparin on the protamine titration.

Normal dog. Blood was drawn from a dog, and 11 ml was added to each of 10 conical centrifuge tubes containing graded amounts of heparin. Beginning with 0.3 mg of heparin the range was increased 0.1 mg per tube and up to 2 mg per 11 cc of blood. Each tube was slowly inverted 20 times to assure mixing. One ml of blood from each tube was allowed to run gently down the sides of each of 10 serology tubes (1.0 x 8.0 cm) containing increasing amounts of protamine sulfate of the same quantity employed in the protamine titration.⁽⁴⁾ These tubes were then shaken individually to mix the heparinized blood with the protamine sulfate. Each rack of 10 tubes was allowed to stand for one hour before the end-point was read. The position of the end-point shifted according to the concentration of heparin. The least amount of heparin found effective in increasing the protamine titration was 20 μ g (1 tube was 18 μ g). This was the equivalent of 6 to 10 times the least effective concentration of heparin required to increase the in vitro clotting time of otherwise unaltered blood (Figure 14).

Toluidine blue titrated against standardly heparinized blood is much less effective in vitro than protamine sulfate. Because different lots of toluidine blue have varied considerably in their antiheparin in vitro activity, this approach was abandoned. Titration of heparin activity with protamine sulfate, however, was found to be satisfactory, and although some variations occurred from lot to lot, this was overcome by standardizing each new lot against a standard heparin preparation. Rosenthal⁽¹¹⁾ has

Figure 12. Effect of intravenous heparin (0.5 mg per kg) on the whole-blood clotting time of normal and irradiated dogs.

- CLOTING TIME OF 1 ML BLOOD MIXED WITH 3 µg HEPARIN
- WHOLE BLOOD CLOTING TIME 15 MIN AFTER I.V. INJECTION OF 0.1 MG/KG HEPARIN
-● WHOLE BLOOD CLOTING TIME ○—○ PLATELET COUNT

Figure 13. Dog 592 received 450 r X irradiation and whole-blood transfusions, 10 ml per kg daily.

Figure 14. Titration of in vitro heparinized blood with protamine sulfate.

likewise failed in attempting to detect heparin or heparinoid material by a toluidine blue titration procedure. He states that "in two leukemic patients with marked hemorrhages, the toluidine blue titration test was negative for free or increased heparin in the blood." The limitation of toluidine blue in such in vitro procedures must be realized, and that the defect described in certain patients with leukemia is merely heparin-like and is but one of the hemostatic disturbances also present. The heparinoid disturbance is certainly not identical with the clotting changes produced by the intravenous injection of commercial bovine heparin.(1,2,6,11,12)

Prothrombin deficiency. Severe prothrombin deficiency increases the amount of protamine required to coagulate a sample of standardly heparinized blood (Figure 15). This is probably a combined effect of both prothrombin deficiency and heparin since neither protamine sulfate nor toluidine blue has the capacity to return the prothrombin time to or toward normal.

Irradiation thrombocytopenia. The amount of protamine required to coagulate standardly heparinized blood in vitro often increases after irradiation.⁽¹⁾ These increases can be returned to normal in some but not all animals with bleeding temporarily, partially, or completely controlled by the administration of toluidine blue. Protamine sulfate is less effective. The response obtained is independent of the platelet level. Consequently, that portion of bleeding due to thrombocytopenia is unchanged and remains untreated. It has been already pointed out that thrombocytopenia may occur with no increase in this type of protamine titration. When bleeding complicates thrombocytopenia when the protamine titration is normal, toluidine blue or protamine sulfate has little or no effect on bleeding.

The relationship between the protamine titration and thrombocytopenia in vitro has been studied in the manner that follows. A series of experiments was conducted in which standardly heparinized whole blood was slowly centrifuged, and the plasma obtained subsequently was centrifuged at high speeds (22,000 G) for 15 minutes at room temperature. This removed nearly all of the platelets. This plasma was re-mixed with the red cell mass and the centrifugation procedures were carried out again. In all, eight of these cycles were required to produce a severely "thrombocytopenic reconstructed blood." This blood was finally made up to the original cell volume. The protamine titration was then performed on this reconstructed blood. The data obtained are presented in Table 14, which shows no change in the protamine titration, performed in triplicate, within the levels

Figure 15. Prothrombin deficiency. Effect on protamine titration and whole-blood clotting time.

Table 14

Protamine titration and platelet counts on reconstructed whole blood

Cell mass washed with isotonic saline		Cell mass washed with its own plasma obtained from standardly heparinized blood	
Protamine titration*	Platelet count per cu mm	Protamine titration	Platelet count per cu mm
0.100	120,000	0.140	140,000
0.100	60,000	0.140	12,000
0.100	30,000	0.140	12,000
0.100	10,000	0.140	12,000
0.100	8,000		
0.100	8,000		

*Following the addition of a standard concentration of heparin to normal blood, a remarkably constant amount of protamine is required to restore clotting. (Laboratory normal is 0.140 mg protamine sulfate (Lilly) per ml blood containing 0.091 mg heparin (Abbott).)

of thrombocytopenia produced. Such an experiment is open to criticism since trauma is produced by the rigorous procedure. However, the results are similar to those obtained from uncomplicated idiopathic thrombocytopenia.

The protamine titration was also performed on "platelet poor plasma" obtained by high speed centrifugation of standardly heparinized normal dog blood. The amount of plasma added to each protamine tube corresponded to the plasma content of 1 ml of whole blood. The results obtained are presented in Table 15. There was a definite increase in the titration values obtained on plasmas with platelet counts less than 80,000 per cu mm. The differences in these data and those obtained on reconstructed thrombocytopenic whole blood were consistent; presumably, this was due to thromboplastin liberation from cellular breakdown incident to the procedures employed.

Table 15

Protamine titrations on plasma with varying platelet counts

Protamine titration*	Platelet counts per cu mm
0.120	348,000
0.120	170,000
0.120	136,000
0.120	95,000
0.120	80,000
0.140	8,000
0.140	8,000
0.140	"None"
0.160	"None"

*Following the addition of a standard concentration of heparin to normal blood, a remarkably constant amount of protamine is required to restore clotting, (Laboratory normal is 0.140 mg protamine sulfate (Lilly) per ml blood containing 0.091 mg heparin (Abbott).)

Effect of in vivo heparin on the protamine titration.

Normal dog. The least amount of intravenous commercial bovine heparin required to increase the protamine titration, as determined 15 min. after heparin injection, was found to be 1.35 to 1.50 mg per kg of body weight (Figure 16). This amount of heparin invariably produced an incoagulable blood, and was 5 to 6 times the minimal quantity required to produce a detectable increase in the whole-blood clotting time in vivo. The amount of intravenous heparin required to increase the titration to 200 μ g ranged between 3.0 and 4.0 mg per kg of body weight.

Irradiation thrombocytopenia. Daily records of the whole-blood clotting time, protamine titration, heparin sensitivity, and platelet count in an irradiated dog are given in Figures 12 and 14. These data indicate that increases in the whole-blood clotting time are far less than would be expected if this measurement were a function of the protamine titration or if the protamine titration were a function of heparin. As indicated

Figure 16. Changes in whole-blood clotting time and protamine titration in a normal dog. Blood samples 10 min. after heparin injection.

in paragraph above, the amount of commercial bovine heparin found necessary to increase the titration one increment ($20 \mu\text{g}$) was 1.35 to 1.50 mg per kg, and this amount invariably resulted in an incoagulable blood. It would seem then, that if the factor(s) responsible for the increases in whole-blood clotting time and the titration in the irradiated dog are due to heparin-like substances, and if the protamine binding property of that substance is equal to that of commercial bovine heparin, its anticoagulant potency is less than that of commercial heparin by several hundred times, if indeed the protamine titration changes observed are the result of an anticoagulant at all.

Prothrombin deficient dog. The effect of in vivo heparin is potentiated in the prothrombin deficient dog when the prothrombin level is below 50 per cent of normal, but the protamine titration end-point clot is not affected until the level of prothrombin activity is 35 to 30 per cent or less.

DISCUSSION

The functions of the platelet in coagulation are multiple and incompletely known. It is established, however, that they activate thromboplastin, agglutinate, and adhere at the sites of vascular injury, and are associated with the syneresis of the fibrin clot. The studies herein presented indicate another potential function of the platelet -- that of antiheparin. Whether this is a physiologic function depends upon whether heparin is a normal constituent of blood. This well may be true for Monkhouse et al.⁽¹⁵⁾ have been able to isolate from normal blood a metachromatic material with low grade antithrombic activity. It also may be significant that certain portions of platelets stain metachromatically with toluidine blue.⁽¹⁶⁾

If the antiheparin action of the platelet functions physiologically, it is possible that in thrombocytopenia there is an increase in "free" heparin unless this is compensated for by other antiheparin mechanisms such as decreased production, an increased destruction or excretion, a decrease in heparin co-factor, or through the formation of other heparin complexes with little or no anticoagulant potency. That some of these factors may be affected to different degrees and in various combinations is suggested by the varied clinical picture and therapeutic response in thrombocytopenia.^(2,17)

The protamine titration is a phenomenon that seems to be independent of the platelet count⁽⁹⁾ and does not necessarily parallel the clotting time. In this respect, the results of the protamine titration resemble those of the antithrombin titer which also do not necessarily forecast an increase in the clotting time.⁽¹⁸⁾ However, if there is any similarity between the mechanism concerned with the protamine titration and the antithrombin titer of Astrup,^(19,20) it is not yet evident from our data.

The increased sensitivity of blood to heparin, in vitro and in vivo, often was associated with an increased protamine titration. This increase, however, was much more closely related to the platelet level than to the titration. This point was well illustrated by the aminopterin-treated dogs in which increases in protamine titration occurred but thrombocytopenia did not develop and heparin sensitivity continued within the normal range. (12)

Disturbances in the clotting mechanism may influence the first or second phases of coagulation -- or both. These disturbances may delay or accelerate the rate of clotting, and both may exist simultaneously. The most frequently encountered accelerators of coagulation are artifacts and usually result from poor technique. Clotting times of less than 20 min. by dry tube methods are usually meaningless and may obscure the presence of important clotting time changes that may be evident with careful technique and skill. This often is illustrated at autopsy in abnormal bleeding cases wherein rapid clotting of blood occurs soon after vessels are severed even though this blood has remained fluid intravascularly for many hours after death.

It is obvious that the greatest need in the study of blood coagulation is the development of better methods for study. The introduction of dry film coatings for equipment represents a real technical advance in this direction, but it cannot be expected to overcome the errors introduced by undue trauma at the site of venepuncture.

SUMMARY

1. In vivo and in vitro heparin sensitivity are greatly increased in thrombocytopenia and prothrombin deficiency.
2. One of the potential functions of the platelet is its limited antiheparin action. It is not known whether this is a physiologic action in continuous operation.
3. A series of experiments is presented that indicate that changes in heparin sensitivity of blood and the protamine titration, also performed on whole blood, are not identical.

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5. AMINOPTERIN IN NORMAL DOGS*

by

B. J. Grossman, M. H. Sanderson, and J. G. Allen

INTRODUCTION

Since both irradiation and nitrogen mustard produce hemorrhage as well as profound changes in the hematopoietic and "reticulo-endothelial" systems,⁽¹⁻³⁾ it seemed possible that 4-aminopteryl glutamic acid (aminopterin) might also induce bleeding.

METHODS

1/ Ten dogs were given intramuscular injections of 0.5 mg of aminopterin daily for varying periods of time that ranged from 4 to 16 days. One dog was given 1.0 mg of aminopterin daily for 7 days and another received a daily dose of 1.5 mg for 3 days.

2/ The following studies were made on each animal: erythrocyte, leucocyte, and thrombocyte counts; differential leucocyte counts; whole-blood clotting times; prothrombin activity;⁽⁴⁾ protamine titrations;⁽⁵⁾ and gross observations for evidence of clot retraction and fibrinolysis.^{1/2} The effect of in vitro added heparin was measured daily on 3 dogs given aminopterin, and daily control values were recorded for a normal animal.

RESULTS

Gross blood was found in the stool of 8 of the 10 dogs. Dogs 441 and 531, which received daily 1.5 and 1.0 mg of aminopterin, respectively, did not develop evidence of hemorrhage. Dog 441 died on the third day of aminopterin treatment and dog 531 died on the 7th day. Of the 10 dogs given 0.5 mg daily, 9 showed external evidence of hemorrhage before death.

When bleeding occurred, the protamine titration was increased in all but 2 dogs. However, increases in the titration were also seen at times when no bleeding was present. In fact, all animals at some time during the experiment showed an increase in the titration whether or not they bled. In some this increase was evanescent and lasted for only one day or less. The greater the increase in the titration, the greater was the incidence of

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bleeding and the more extensive were the hemorrhagic manifestations. However, in no instance did hemorrhage appear responsible for the death of an animal although when aminopterin was continued, all animals died (Table 16).

Five of the 10 dogs were treated with 5 mg per kg of toluidine blue. Except for intestinal bleeding, all other evidence of hemorrhage stopped within 12 hr. The platelet counts showed no consistent pattern and, in general, were not appreciably altered. Dogs 513 and 538 are representative of the group in this respect (Figures 17 and 18).

Prothrombin activity gave no appreciable evidence of decrease save for two incidences when a slight decline occurred on the last day of life. No evident change was observed in fibrinolytic activity or clot retraction.

Contributing to bleeding were the hyperemia and desquamation of the gastro-intestinal tract. Microscopically, the mucosa is markedly distorted by superficial erosion, inflammation, edema, metaplasia, and hemorrhage (Figure 19). The mucosal glands were shortened and irregular compared with the normal mucosa (Figure 20). These changes were similar to those previously described by Farber.⁽⁶⁾

In three animals the effect of added heparin on the in vitro whole-blood clotting time was determined daily and was compared with similar data obtained daily on a normal control dog. No consistent differences were observed (Figure 21). This is in sharp contrast to the increased heparin sensitivity seen in the irradiated animal, which develops not only an increase in the protamine titration but, in addition, severe thrombocytopenia.

DISCUSSION

These² observations disclosed at least two possible explanations for the hemorrhage occurring in the normal dog given aminopterin. One was a general effect that was manifested by an increase in the protamine titration and frequently by a mildly prolonged clotting time. The defect(s) seemed amenable to the administration of adequate amounts of toluidine blue and/or protamine sulfate. The second was a local effect in which capillary denudation occurred as a result of gastro-intestinal desquamation.^{2/3} The animals given toluidine blue showed a diminution or complete cessation of bleeding about the gingival margin if ulcerations were not also present. Gastro-intestinal bleeding, however, was not materially affected. The latter appeared largely local in origin and stopped spontaneously 3 or 4 days after withdrawal of aminopterin. Cessation of gastro-intestinal bleeding in these animals probably depended to a large extent upon the healing of local areas.

Table 16

Summary data of dogs given aminopterin

Dog number	Wt. (kg)	Aminopterin (mg)			Day of bleeding	Remarks
		Daily dose	Total dose before bleeding	Total dose		
528	8.5	0.5	5.0	5.5	9	Died on 10th day.
538	8.5	0.5	6.5	7.5	13	Aminopterin stopped on 14th day. Given toluidine blue on 14th day. Recovered.
513	9.7	0.5	6.5	8.5	13	Died on 16th day. Given toluidine blue on 15th day.
836	8	0.5	4.5	5.5	8	Died on 10th day.
188	9	0.5	2.5	10.0	5	Aminopterin discontinued from 5th to 11th day. Then re-administered daily until death on 19th day. Bleeding did not recur.
699	10	0.5	2.0	3.0	4	Died on 5th day.
460	10	0.5	8.5	8.5	-	Received aminopterin daily until 16th day. No bleeding.
959	14.3	0.5	4.0	5.5	9	Given toluidine blue on 10th day. Bleeding stopped 5 hr later. Did not recur next day. Aminopterin stopped following day. Given 150 mg folic acid. Recovered.
960	16.5	0.5	3.5	5.0	7	Given 5 mg/kg toluidine blue. Bleeding stopped 8 hr later. Bleed- ing recurred slightly next day. Animal died.
983	8	0.5	1.5	2.0	3	Toluidine blue given 3rd day. Bleeding decreased 4 hr later. Died next morning. No evidence of oral bleeding.
531	9	1.0		8.0	-	Died on 7th day.
441	9	1.5		4.5	-	Died on 3rd day.

Figure 17. Effect of toluidine blue on dog treated daily with 0.5 mg aminopterin (dog no. 513).

Figure 18. Effect of toluidine blue on dog treated daily with 0.5 mg aminopterin (dog no. 538).

Figure 19. Mucosa of the lower small intestine of aminopterin-treated dog.

Figure 20. Mucosa of the lower small intestine of a normal dog.

Figure 21. In vitro heparin sensitivity (dog no. 983).

Two other points illustrated by these experiments were that the protamine titration may be increased without thrombocytopenia or prothrombin deficiency and that in vivo heparin sensitivity was not materially altered when the protamine titration was increased.

If these findings are applicable to the leukemic patient receiving aminopterin, it may be that the administration of large doses of aminopterin would contribute to hemorrhage. If so, this is in addition to the thrombocytopenia frequently present in the leukemic patient. Platelet thrombopenia can be expected to seriously affect bleeding in ulcerated areas of the type seen in the gastro-intestinal tract after aminopterin, and such bleeding will occur and is not influenced by toluidine blue and/or protamine sulfate.

3/ It is generally agreed that the platelet thrombus is an important hemostatic mechanism that acts primarily at the site of vascular injury. It is likely that the thrombocytopenic leukemic patient who receives aminopterin and who develops mucosal desquamation in the gastro-intestinal tract will bleed more profusely than if the platelet count is normal. Many of these thrombocytopenic leukemic patients also show an increase in the protamine titration whether or not they receive aminopterin. This latter defect usually can be corrected or improved by toluidine blue administration with some control of peripheral bleeding. Since toluidine blue does not influence the platelet count in these patients and it is not a substitute for platelets and will not influence hemorrhagic manifestations secondary to thrombocytopenia alone, the control of hemorrhage in some thrombocytopenic leukemic patients on aminopterin therapy may present a difficult problem.

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6. THE ANTIHEPARIN ACTIVITIES OF PROTAMINE SULFATE, GELATIN, LYSINE, AND TOLUIDINE BLUE*

by

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INTRODUCTION

One of the characteristics of heparin is that it forms dissociable compounds with a number of natural and synthetic substances. Many of these substances are positively charged and basic and contain free amino groups. Heparin itself is strongly acid and bears a negative charge. These complexes of heparin may or may not be devoid of anticoagulant activity. This seems to be determined by whether the affinity of the particular substance for heparin is greater or less than that of the blood proteins, etc., through which heparin exerts its anticoagulant action. Many proteins, amino acids, and dyes⁽¹⁾ combine with heparin. Some of these combinations are strongly bound and nullify the anticoagulant action of heparin. The firmness of the bond cannot be predicted from in vitro work, as an agent may have little or no in vitro antiheparin activity and yet display definite in vivo antiheparin action. When present in sufficient concentration, some of these antiheparins develop anticoagulant activity. Because of the possible therapeutic value of the antiheparins in the control of certain hemorrhagic states, a series of experiments was undertaken to determine which of several antiheparins was most suitable from the standpoint of human therapy.

METHODS

All experiments herein reported were conducted on the blood of dogs. Measurements included whole-blood clotting time,⁽¹⁾ protamine titration,⁽²⁾ prothrombin time,⁽³⁾ gross observation for clot retraction and lysis, and routine hemocytology.⁽⁴⁾

The antiheparin agents investigated included toluidine blue (Abbott), protamine sulfate (Lilly), gelatin (Winthrop-Stearns), and dl lysine (Merck). In each instance Abbott liquid commercial bovine heparin was used.

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EXPERIMENTAL

The total length of time after the intravenous injection of 0.5 mg heparin per kg of body weight during which increases in the whole-blood clotting time can be detected in normal dogs is fairly constant -- approximately 90 min.⁽¹⁾ Because of this, it is possible to inject heparin at different intervals after antiheparins are given and to detect whether any residual effective antiheparin action is present. In addition, the character of the plotted time curve of clotting times is important in studying the effect of the antiheparins, especially when these are normal at the 90-min. postinjection period.

Protamine sulfate: Protamine sulfate is a potent antiheparin that acts almost instantaneously and is the most effective agent yet available in treating over-heparinization.⁽¹⁾ Its duration of detectable action is relatively brief, probably less than 2 hr. When heparin is given intravenously to normal dogs and followed by an equal weight of protamine sulfate, also given intravenously, the protamine effect may disappear more rapidly than that of heparin. This is evident only when the doses of protamine and heparin are 1 mg each or greater. The results obtained when 2 mg of protamine sulfate per kg was given 15 min. after 2 mg of heparin are shown in Figure 22. The clotting time returned to normal, but 45 min. later was slowly increased and did not again return to normal until 1 1/2 hr later. When the doses of heparin and protamine sulfate were 0.5 mg per kg each, the effects of both of these substances were spent at approximately the same rate, and no secondary increase in the clotting time occurred.

Similar data were obtained by injecting protamine sulfate at various intervals prior to heparin administration. In this way it was possible to determine how long protective action against heparin was afforded by protamine. These data, presented in Figure 23, show that no protection was evident 90 min. after protamine injection.

The fate of injected protamine is unknown. It may be removed from the blood stream and later metabolized or excreted. It also is possible that protamine is rapidly destroyed by enzymatic activity since protaminease is known to be abundantly present in plasma.⁽⁵⁾

Ox polygelatin: This material displayed only slight in vitro anti-heparin action. In fact, its in vitro activity could be easily overlooked. When given intravenously, however, it had an antiheparin activity nearly as potent as that of protamine sulfate (Figure 24).

The duration of gelatin protection as determined by the subsequent injection of heparin was greater than that of protamine sulfate on similar mg per kg body weight dosage levels (Figure 25). When 1 g of gelatin per kg of body weight was administered, the results obtained were unexpected.

Figure 22. Duration of protamine activity and its relation to various levels of intravenous heparinization in dogs (Oct. 1948).

Figure 23. Effect of prior injection of protamine sulfate on the whole-blood clotting time with in vivo heparinization.

Figure 24. Effect of intravenous administration of heparin followed by intravenous administration of gelatin.

Figure 25. Normal dog clotting time response to 0.5 mg/kg heparin after previous injection of 2 mg/kg gelatin.

There was no protective action against heparin unless more than 1 hr elapsed before heparin was given (Figure 26). Thereafter, as the time lapse increased, the antiheparin action of this agent became more apparent for 4 hr. A similar experiment could not be performed with large amounts of protamine because of the anaphylactoid reaction produced by the amounts required. This delayed protective action suggests that there is an optimal point of concentration of an optimal gelatin-heparin ratio before a satisfactory antiheparin effect is obtained. It may be that heparin inhibition is not manifested until the excess quantities of gelatin are removed from the blood stream. The exact time that the antiheparin action was exhausted was not determined although none could be detected 18 hr after gelatin injection.

Lysine: Jacobson and Farber⁽⁶⁾ have used this amino acid with success in the treatment of bleeding in the leukemic patient whose protamine titration is increased. The rationale of its use was based on the observation that the more common and effective antiheparins are basic and have free NH_2 groups. Lysine, like gelatin, has essentially no in vitro antiheparin action. On the other hand, this amino acid is a moderately effective antiheparin in vivo (Figure 27). Of more practical interest is the moderate antiheparin action lysine affords when given orally. This is illustrated in a series of charts composing Figure 28. Apparently there is a cumulative action since 2 or 3 days of administration were necessary to achieve maximum protection. Its antiheparin action disappears in less than 30 hr after the last oral dose of lysine is given.

Lysine does not have any in vitro anticoagulant action in concentrations up to 4 mg per ml of blood (Table 17).

Table 17

In vitro antiheparin and anticoagulant relationships of protamine sulfate, toluidine blue, ox polygelatin, and dl lysine

	<u>Unaltered blood</u>	<u>Heparinized blood, 0.091/ml</u>	
	Anticoag. action in $\mu\text{g}/\text{ml}$	Antiheparin action in $\mu\text{g}/\text{ml}$	Anticoag. action in heparinized blood in $\mu\text{g}/\text{ml}$
Protamine sulfate	160 or more	140	1000
Toluidine blue	300 or more	200	8000
Ox polygelatin	none(up to 18,000)	8400	none(up to 10,000)
dl Lysine	none(up to 4000)	none(up to 4000)	none(up to 4000)

Figure 26. The effect of intravenous heparinization in dogs at different time intervals following the administration of gelatin (Dec. 1948).

Figure 27. Effect of lysine following intravenous administration of 0.5 mg/kg heparin.

Figure 28. Effect of orally administered lysine on the whole-blood clotting time following administration of 0.5 mg/kg heparin.

The protective action of lysine against the subsequent injection of heparin lasts longer than that of protamine and was similar to that of gelatin. Data obtained from dogs given intravenous injections of lysine (2 mg per kg) at intervals ranging from 15 min. to 2 hr prior to the intravenous injection of 0.5 mg heparin per kg are shown in Figure 29.

Toluidine blue: This dye has a mild but slow antiheparin action that can be detected both in vitro and in vivo. Its action is not readily evident, and not all investigators are agreed that this phenomenon occurs. This is probably because its action is slow compared to the nearly instantaneous action of protamine. The slow reaction of toluidine blue with heparin can be demonstrated both in vivo and in vitro (Figures 30 and 31). However, toluidine blue resembles protamine sulfate in that if given in excess it acts as an anticoagulant. In this respect, toluidine blue and protamine sulfate differ from both lysine and gelatin (Table 18). Anti-heparin activity following a single intravenous injection of toluidine blue (5 mg per kg) can be detected for 24 to 30 hr (Figure 32).

Table 18

Heparin-antiheparin relationships

	<u>In vitro</u> mg/ml blood	<u>In vivo</u> mg/kg body weight
Protamine-heparinate	0.140 - 0.091	0.25 - 0.50
Toluidine blue - heparinate	0.200 - 0.091	1.00 - 0.50
Gelatin-heparinate	8.400 - 0.091	1.00 - 0.50
Lysine-heparinate	Unknown	

Since toluidine blue is a strongly hemolytic agent for the dog, it seemed possible that hemolysis might contribute to its antiheparin action. To check this possibility acute hemolysis was produced by the rapid intravenous injection of 200 ml of distilled water. Heparin was injected before and twice after the water injection (Figure 33). A slight decreased heparin effect was detected an hour after the injection of water but none was evident 24 hr later.

The dye is rapidly decolorized in the plasma, and in the therapeutic range in dogs no color persists 10 min. after injection. The "leuco" form, however, appears to be active as an antiheparin. It is probable that all experiments outlined above in which toluidine blue was administered prior to heparin depended upon the antiheparin action of the leuco rather than the

Figure 29. Normal dog whole-blood clotting time response to 0.5 mg/kg heparin after injections of lysine.

Figure 30. Effect of prior injection of toluidine blue on whole-blood clotting time with in vivo heparinization.

Figure 31. Titration of toluidine blue and protamine sulfate against standardly heparinized normal dog blood. In vitro clotting end-point readings at various intervals after treatment.

Figure 32. Effect of prior injection of toluidine blue on whole-blood clotting time with in vivo heparinization.

Figure 33. Effect of heparin on water-induced hemolysis.

blue form of the dye. Plasma in vitro will decolorize about 2 to 5 μg per ml whereas in vivo this capacity is increased to about 100 μg per ml. The blue form may be present in the urine for several days after it has disappeared from the plasma.

It is known that both toluidine blue and protamine sulfate are anticoagulants as well as antiheparins. A series of observations were conducted in an attempt to determine the concentrations of protamine sulfate, toluidine blue, ox polygelatin, and dl lysine at which anticoagulant activity occurred. Similar studies were made on heparinized blood (0.091 μg per ml blood). The results of these experiments are shown in Table 18. Neither gelatin nor lysine displayed any anticoagulant effect in doses not in excess of their therapeutic effect. Both toluidine blue and protamine sulfate, however, displayed anticoagulant activity in concentrations exceeding those required for their antiheparin activity.

DISCUSSION

Protamine sulfate and toluidine blue are both potent antiheparins, but in radiation hemorrhage therapy, protamine has been found to be less effective than toluidine blue. These experiments offer one possible explanation -- the duration of protamine effect is brief, less than 2 hr. Toluidine blue has an effect for 12 to 24 hr. When given in large amounts protamine produces an anaphylactoid state, thus limiting its usage.

Toluidine blue is unphysiologic and it should be replaced as soon as an effective physiologic compound becomes available. These preliminary experiments indicate that lysine does not meet these requirements completely but it may prove a suitable adjuvant. Of particular interest is the fact that lysine has some limited value when administered orally as well as parenterally.

A great deal of attention has been given to the fact that heparin does form salts although little emphasis has been placed upon the physical and biochemical nature of the salts formed. The research of Fischer⁽⁷⁾ has clearly shown these reactions to be reversible and that they follow the mass action law. One of the salts tested was thromboplastin-heparinate which Fischer and Astrup⁽⁸⁾ found to behave in accordance with the mass law. The large number of proteins with which heparin can form salts raises the question whether this agent is normally free to any great extent in blood. Presumably the percentage of total heparin that is free at any one time would depend upon the dissociation constants of the heparin-protein complexes current at the time of study. As is the case with most of the simple salts formed by the union of weak acids and weak bases, there is also a dissociation or ionization constant for heparin-proteinate complexes. For the heparin-thromboplastin complex, this is essentially a

stoichiometric relationship. Presumably, this relationship also holds for other heparin-protein salts; i.e.,

$$\text{From the mass law, } \frac{C_{\text{heparin}} \times C_{\text{protein}}}{C_{\text{heparin-proteinate}}} = K.$$

Then, if $\underline{a}$ is the concentration of added protein, $\underline{c}$ the concentration of free protein, and $\underline{b}$ that of added heparin, $\underline{a} - \underline{c}$ is the concentration of the product formed by the reaction, $\underline{b} - (\underline{a} - \underline{c})$ is the concentration of free heparin, and

$$K = \frac{\underline{c} \underline{b} - (\underline{a} - \underline{c})}{\underline{a} - \underline{c}}$$

Since K must remain constant, a change in terms of one of the factors in the above equation must be compensated for by a change in the others. An increase in heparin-proteinate is accompanied by an increase in the undissociated heparin-proteinate and vice versa.

It is our observation that the increased heparin sensitivity in thrombocytopenia appears to be a function of dissociation which is brought about by a decline in the antiheparin factors concerned with platelet numbers. This reaction also is in part a function of the concentration of the constituents which may not be completely dissociated except in dilute solutions. In this respect, the observations of Morawitz⁽⁹⁾ are of interest, with respect to the anticoagulant action of heparin. He observed that by simple dilution the effect of the anticoagulant liberated in peptone shock could be completely annulled. We have confirmed this finding in the blood of animals given heparin, in some animals with prolonged coagulation times after X irradiation and in a few patients with "heparinoid" bleeding disorders⁽¹⁰⁾ if an anticoagulant is demonstrated.

SUMMARY

1. Antiheparin activity may be detected for protamine sulfate, toluidine blue, ox polygelatin, and lysine. This can be demonstrated in vitro and in vivo with protamine and toluidine blue but only in vivo with gelatin and lysine.

2. The antiheparin action of protamine sulfate, while prompt and effective, lasts for less than 2 hr while that of toluidine blue is slow but lasts from 12 to 24 hr. Gelatin and lysine last 12 to 24 hr. The rapid inactivation of protamine probably explains why this substance is less effective than toluidine blue in the treatment of "heparinoid" bleeding.

3. The amino acid -- dl lysine -- is a mild antiheparin when given orally and parenterally. It is more physiologic than toluidine blue and it is less toxic than protamine sulfate. (auth) 10 references. 1/END

* * *

CB 1-19-51

Treatment of the hemorrhagic disorder associated with irradiation and various other disorders with toluidine blue and protamine sulfate has been described elsewhere.⁽¹⁰⁾

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7. PLASMA ANTITHROMBIN IN IRRADIATED AND HEPARINIZED DOGS

A PRELIMINARY STUDY*

by

D. M. Enerson, B. J. Grossman, and J. G. Allen

INTRODUCTION

The inactivation of thrombin by serum was recognized by Schmidt in 1892.⁽¹⁾ The term antithrombin was first used by Morawitz⁽²⁾ in 1904 to describe a substance present in normal serum that inactivated thrombin. Since then this term has been used to describe any substance, synthetic or natural, that inhibits thrombin activity. Some of these substances function competitively, and when the affinity of one for thrombin exceeds that existing between thrombin and fibrinogen, coagulation is retarded or fails to occur. Such agents include cysteine, hirudin, and heparin when in the presence of plasma co-factor. In addition, thrombin is apparently adsorbed by certain plasma proteins with the result that thrombin is no longer available for coagulation.

This section is concerned with measurements of plasma antithrombic activity in normal dogs, in dogs given heparin, and in X-irradiated dogs. Since heparin is antithrombic in the presence of plasma co-factor,⁽³⁾ it was thought that knowledge of plasma antithrombic titers might help to clarify the relationship between heparin and the heparinoid abnormality of the irradiation syndrome.

METHODS

Coagulation studies included the whole-blood clotting time,⁽⁴⁾ prothrombin time,⁽⁵⁾ the protamine titration,⁽⁶⁾ gross evidence for lysis and clot retraction, complete hemocytology, including platelet counts, and plasma protein determination.

Antithrombin titers were estimated by the method of Astrup and Darling.⁽⁷⁾ Venous blood was oxalated with 1/7 volume of 1.84 per cent potassium oxalate in clean dry glassware. Plasma was removed after 20 min. of centrifugation at 2000 rpm. Antithrombin estimation was determined on four samples of the same plasma (0.05, 0.10, 0.15, and 0.20 ml). Each of these tubes was made up to 0.30-ml volume with normal saline. To

*This work was supported by the Medical Department, U. S. Army, the Biological and Medical Research Division of the Argonne National Laboratory, and the Department of Surgery of the University of Chicago.

each of these samples was added 1 ml of thrombin (Parke-Davis) of 45.5 units potency. Each tube was then incubated 15 min. at 37°C. One ml of a 0.2 per cent bovine fibrinogen (Armour) solution was added to 0.10 ml of this mixture after incubation, and the time necessary for clot formation was noted. Each of these fibrinogen clotting times was done in duplicate, and the results were averaged.

With each series of 4 samples, a control sample was incubated without plasma. It contained 1 ml of thrombin solution with 0.30 ml of normal saline. One ml of the fibrinogen solution was added to 0.10 ml of this mixture and the time for fibrin appearance was observed.

The fibrinogen clotting times of the antithrombin material (plasma) were converted into antithrombin units by the calculations of Astrup and Darling.⁽⁷⁾

Only two slight modifications of the original method were employed in our study. Thrombin was not filtered nor centrifuged since this material made a water-clear solution and with these reagents it was not necessary to add octyl alcohol to prevent foaming.

The method for measurement of antithrombin levels was originally worked out on rabbit serum and plasma. In the normal rabbit, the variations from animal to animal and from day to day do not exceed 10 per cent. Relatively little work was done on dogs by Astrup, Darling, and Volkert. Astrup, in commenting on the determination, mentioned its variability when working with dog plasma or serum. This has been true in our work. Although in most of the animals the values obtained during the control periods were generally reproducible, there were variations from day to day. All references to increases or decreases in antithrombin levels in this report refer to changes more pronounced than the variations observed during the control period for that particular dog.

EXPERIMENTAL

Normal dogs: Antithrombin titers were performed on 4 normal dogs prior to and at 15-min. intervals after the intravenous injection of heparin 0.50 mg per kg of body weight. The results obtained are shown in Figure 34 along with the changes in the whole-blood clotting time. The antithrombin titer increased only slightly in contrast to sharp increases in the whole-blood clotting time. In all of these animals there was a secondary and unexplained rise in antithrombin titer even though the whole-blood clotting time had returned to normal (Table 19).

Irradiated dogs: These studies were carried out on 5 X-irradiated dogs that received no treatment and on 12 X-irradiated dogs that were given transfusions daily throughout the postirradiation period. All animals received 450 r total-body X irradiation.

Figure 34. Antithrombin titers for 4 normal dogs prior to and after the intravenous injection of 0.50 mg per kg heparin.

Table 19

Antithrombin and clotting studies on heparinized dogs

(0.5 mg per kg heparin given each animal I.V.)

	Pre-heparin	Min. after heparin					
		15	30	60	90	120	150
Dog 179							
Clotting time (in min.)	28	124	82	56	40	25	23
Protamine titration (μ g)	120	--	--	--	--	--	--
Antithrombin units	45	61	52	52	27	45	52
Dog 193							
Clotting time (in min.)	29	138	89	60	41	30	28
Protamine titration (μ g)	120	--	--	--	--	--	--
Antithrombin units	42	48	52	33	27	45	27
Dog 366							
Clotting time (in min.)	28	118	73	40	23	25	20
Protamine titration (μ g)	120	120	120	120	120	120	120
Antithrombin units	55	109	61	61	55	61	80
Dog 66							
Clotting time (in min.)	26	110	60	40	30	28	20
Protamine titration (μ g)	120	120	120	120	120	120	120
Antithrombin units	27	66	30	36	36	42	52

Three of the 5 untreated irradiated dogs had a rise in antithrombin titer (Figure 35 and Table 20). These increases occurred at the same time that the changes in the whole-blood clotting time appeared.

The 12 that were given transfusions all showed similar rises in antithrombin titer, which also progressively increased during the postirradiation period. The whole-blood clotting times were increased, but, as in the case of the 5 untreated irradiated animals, the clotting time increases were minor compared with those obtained in the heparinized normal dogs (Figure 36 and Table 21).

DISCUSSION

Our observations confirm those of Astrup and Darling⁽⁷⁾ in that the administration of moderate amounts of heparin did not produce a marked increase in antithrombin titer. The situation was reversed in the irradiated animals in which pronounced increases in the antithrombin titers were observed, whereas only moderate increases in the whole-blood clotting time occurred.

The nature of plasma antithrombin is not fully known. Astrup and co-workers believe it is comprised of two components. One component is thermolabile and is said to be closely bound to the albumins. The other is believed to be similar to heparin plus co-factor, is thermostable, and can be extracted from liver and lungs.

The role of heparin in the antithrombic activity of normal plasma is probably small since the capacity of serum or plasma to inactivate thrombin is not greatly augmented by the addition of heparin. Our data confirm this point of view. It seems likely that the antithrombin titration of Astrup and Darling is a measure of thrombin inactivating substances other than heparin itself, or at least as we know heparin in the highly purified state. This point was well illustrated by Dyckerhoff *et al.*⁽⁸⁾ who were unable to demonstrate any increase in clotting time in guinea pigs and rabbits during immunization or anaphylaxis in spite of the fact that a rise in the antithrombin titer was observed. This has also been noted by Mellanby,⁽⁹⁾ Quick,⁽¹⁰⁾ Volkert,⁽¹¹⁾ and by this laboratory.

Our own experience with antithrombin titers suggests that in the X-irradiated dog they are increased out of proportion to what might be expected from the clotting time increases noted. A similar situation also was observed for the protamine titration after irradiation. In some animals the protamine titration was increased far out of proportion to increases in the whole-blood clotting time. The question arises: does the antithrombin titration of Astrup and Darling measure the same coagulation defect as that measured by protamine titration? Work has not yet progressed to a point where this question can be answered.

Figure 35. Antithrombin titers for X-irradiated dog given 10 ml per kg whole blood daily during postirradiation period.

Table 20

Antithrombin and clotting studies on X-irradiated dogs

	Days pre-irradiation			Days postirradiation													
	3	2	1	0	1	2	3	4	5	6	7	8	9	10	11	12	13
Dog 270																	
WBCT	-	-	33	20	35	28	32	45	41	42	39	58	58	27	36	40	73
P.T.	-	-	120	120	140	120	120	120	140	140	140	120	140	140	140	120	120
ATU	-	-	72	45	48	61	67	27	79	73	91	100	91	108	56	114	97
Dog 112																	
WBCT	-	-	26	-	45	40	33	40	40	41	38	75	100				
P.T.	-	-	120	-	120	120	120	120	120	120	140	120	120				
ATU	-	-	33	-	12	21	36	51	48	36	36	45	90				
Dog 93																	
WBCT	-	-	18	-	40	36	30	30	25	48	38						
P.T.	-	-	120	0	120	120	120	120	120	120	140						
ATU	-	-	36	-	36	21	42	48	36	72	57						
Dog 931																	
WBCT	-	30	26	60	65	30	30	32	45	50	38	35	33	43	62	17	
P.T.	-	140	140	120	140	120	120	120	140	120	120	120	120	120	120	140	
ATU	-	36	30	57	39	57	18	30	72	63	57	57	48	66	66	66	
Dog 832																	
WBCT	-	31	22	43	50	35	35	35	35	40	43						
P.T.	-	120	120	140	120	120	120	120	120	120	140						
ATU	-	60	63	33	27	36	36	27	15	45	45						

Note: WBCT -- Whole blood clotting time in min.

P.T. -- Protamine titration in μg .

ATU -- Antithrombin units.

Figure 36. Antithrombin titers for X-irradiated heparinized dog.

Table 21
Antithrombin and clotting studies on X-irradiated dogs receiving
10 ml per kg whole blood daily

	Days pre-irradiation			Days postirradiation													
	3	2	1	0	1	2	3	4	5	6	7	8	9	10	11	12	13
Dog 236																	
WBCT	24	37	44	-	45	43	34	44	35	41	40	40	37	50			
P.T.	120	120	140	-	120	120	120	140	140	120	120	120	120	120			
ATU	66	99	63	-	69	66	54	115	69	78	87	99	106	121			
Dog 367																	
WBCT	33	-	33	-	40	33	33	34	39	36	35	37	40	46	48		
P.T.	120	-	120	-	120	120	120	120	140	120	120	120	120	120	120		
ATU	57	-	60	-	48	51	33	63	69	148	96	60	90	84	81		
Dog 150																	
WBCT	34	-	32	-	35	44	52	94	80	76	64	102	115				
P.T.	120	-	120	-	120	120	140	140	140	140	140	120	140				
ATU	42	-	-	-	39	45	48	75	57	51	45	90	90				
Dog 702																	
WBCT	36	-	-	-	30	40	51	112	93	160							
P.T.	140	-	-	-	120	140	140	120	140	120							
ATU	30	-	-	-	69	63	75	78	66	87							
Dog 105																	
WBCT	-	23	36	-	40	35	22	45	38	38	30	50	25				
P.T.	-	120	120	-	120	120	120	120	120	120	140	120	120				
ATU	-	42	51	-	30	60	-	63	69	48	75	63	103				
Dog 174																	
WBCT	-	-	25	39	-	25	30	40	33	29	21	50	58	55	44		
P.T.	-	-	120	120	-	120	120	120	120	120	120	120	120	140	120		
ATU	-	-	54	60	-	39	27	66	48	60	66	72	109	87	84		

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Table 21 (Cont'd.)

	Days pre-irradiation			Days postirradiation													
	3	2	1	0	1	2	3	4	5	6	7	8	9	10	11	12	13
Dog 132																	
WBCT	37	33	28	-	37	37	39										
P.T.	120	120	120	-	120	120	120										
ATU	54	24	63	-	72	66	75										
Dog 149																	
WBCT	40	34	-	-	32	31	36										
P.T.	140	120	-	-	120	120	120										
ATU	27	39	54	-	60	60	84										
Dog 364																	
WBCT	34	32	32	-	40	45	44	37	33	55	35	56	59	60	70	42	47
P.T.	120	120	140	-	120	140	140	140	120	120	120	120	140	120	120	120	120
ATU	45	64	45	-	42	42	33	64	66	73	77	85	77	85	85	88	91
Dog 778																	
WBCT	32	24	38	-	43	45	28	36	28	50	43	56	68	42	58	45	26
P.T.	120	120	120	-	120	120	140	120	120	120	120	120	120	120	120	120	120
ATU	63	36	78	-	36	69	106	66	84	69	81	54	103	60	87	78	75
Dog 247																	
WBCT	20	16	21	-	28	59	37	52	47	92	57						
P.T.	140	140	120	-	120	140	120	120	120	140	120						
ATU	78	78	81	-	66	54	106	115	127	133	72						
Dog 312																	
WBCT	36	32	37	-	37	44	40	57	36	43	51	60	53	52	50	48	
P.T.	140	140	120	-	120	120	140	120	120	120	120	140	120	140	120	120	
ATU	63	60	78	-	81	93	81	66	66	87	121	75	90	75	81	69	

As stated in the second paragraph, it was the purpose of this paper to determine whether antithrombin titers increase after irradiation; and if so, does this imply the presence of an increased amount of circulating heparin? While increased antithrombin titers occurred in 15 of 17 X-irradiated dogs, these results were different from those observed after heparin injection because with heparin, the increases in the whole-blood clotting times were greater while the increases in antithrombin titers were less pronounced. When some means have been devised whereby the antithrombin components can be broken down and the components identified and measured, the question of any relationship to heparin probably can be answered.

SUMMARY

1. The blood of 4 normal dogs given intravenous injections of heparin (0.5 mg per kg of body weight) displayed a pronounced increase in the whole-blood clotting time with only a slight increase in antithrombin titer.
2. Fifteen of 17 dogs given total-body X irradiation developed mild to moderate increases in the whole-blood clotting times with pronounced increases in the antithrombin titer.

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8. PHYSICAL PHENOMENA IN BLOOD COAGULATION*

by

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P. V. Moulder, and J. G. Allen

INTRODUCTION

There are many factors that determine whether blood will remain fluid or will clot. Biochemical and enzymatic aspects of blood coagulation have been extensively studied, while physicochemical aspects have been investigated to a lesser extent. The clotting time of blood in vitro depends in part on the wetting characteristics of the surface of the solid with which it is in contact. Generally, the less the degree of wetting of the solid by blood, the longer the clotting time. Thus, containers coated with paraffin, silicone, and other hydrophobic materials are "not wet" by blood, platelets are not readily influenced by the foreign surface, they rupture more slowly, with the result that the rate of release of thromboplastin or thromboplastin precursors is retarded and hence clotting proceeds slowly. Figure 37 demonstrates the difference in wettable qualities of glass by blood and of a paraffin surface by blood.

The degree of wetting of a solid by a liquid can be expressed in terms of the angle of contact between liquid and solid phases. The smaller the angle of contact, the greater the wetting of the solid surface by the liquid. This is exemplified by water wetting glass. On the other hand, when the angle of contact is great, wetting is less, as when water contacts a paraffined surface. These wetting characteristics depend on the surface tensions of the solids and of the liquids that are in contact. Paraffin has a lower surface tension than glass and, therefore, the liquid surface does not spread over it as it does over glass (assuming a constant liquid phase). The small angle of contact and great wetting that occur between ether and glass depend on the low surface tension of the ether while the large angle of contact and little wetting of glass by mercury are determined by the high surface tension of the mercury. This can be explained mathematically as follows: (See Figure 38)

- S_1 = Surface tension of solid; S_2 = Surface tension of liquid;
- S_{1-2} = Interfacial tension solid-liquid
- If $S_1 > S_2 - S_{1-2}$ wetting occurs
- If $S_1 < S_2 - S_{1-2}$ no wetting occurs

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Figure 37. Differences in angle of contact of whole blood on paraffin (left) and on glass (right).

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VARIOUS LIQUID-SOLID CONTACT ANGLES

$S_1 > S_2 + S_{1-2}$ WETTING
 $S_1 < S_2 + S_{1-2}$ NO WETTING

Figure 38. Degree of wetting of solid by liquid as revealed by angle of contact.

It follows that if clotting time in vitro is influenced by the nature of the solid surface, it should also be influenced by the surface tension of the liquid phase, that is, the blood, and also by the nature of the in vivo surface with which blood comes into contact, that is, the intimal lining of blood vessels.

Plasma proteins form the bulk of the colloidal plasma and are surface active.⁽¹⁾ Generally, when the protein concentration is high, the surface tension is low. Moreover, the albumin fraction is probably more important than the globulin fraction, since the albumin molecule is smaller and, therefore, for a given mass of protein, the number of surface active particles is greater for albumin than for globulin. Bottazzi⁽¹⁾ has clearly shown that the surface activity of proteins is related to their particulate nature. Molecular protein is the most surface active. Protein in an ionic state or aggregates of protein become greatly hydrated, and these hydrated particles are less surface active than molecular protein, thus lowering surface tension to a much smaller degree.

This is a preliminary study of the colloidal system of blood plasma with respect to its surface tension, in the nature of the surfaces with which blood comes into contact both in vitro and in vivo, and the influence which these factors have on coagulation of blood.

METHODS

Surface tension: The force (expressed as dynes per cm) required to pull a platinum ring of known circumference through the surface of standardly heparinized plasma (0.091 mg heparin per ml of blood) was determined by means of the Christian-Becker chainomatic surface tension balance. Measurements were made at room temperature within 4 hr after the blood was drawn and the plasma was thoroughly stirred before each reading. The humidity of the surrounding air was not controlled.

Angle of contact of liquid-solid systems: Droplets of various liquids less than 1 mm in diameter were placed on various surfaces from finely drawn glass capillaries. Gross observations and photographs of the angle of contact between the liquid and solid were then made. These, too, were made at room temperature with no control over humidity.

Viscosity: The viscosity (relative to water) of standardly heparinized plasma (0.091 mg heparin per ml of blood) was measured using a Hellige Viscometer, which is based on a flowmeter principle.

Whole-blood clotting time

Protamine titration

Prothrombin activity

Plasma proteins, albumin and globulin, were determined by a semi-macro Kjeldahl method

Routine hemocytology including chamber platelet counts

EXPERIMENTAL

Coagulation studies: Clotting time changes appeared in all of the X-irradiated animals, most of the prolongations occurring during the latter half of the postirradiation period. The platelet count usually fell to levels of 80,000 to 150,000 per cu mm by the 8th postirradiation day. The prothrombin time remained normal, except for a slight drop terminally, and the protamine titration became elevated, usually in the last 2 or 3 days of life.

Plasma surface tension: In 20 normal dogs, the surface tension of the plasma (of standardly heparinized blood - 0.091 mg heparin per ml blood) ranged between 53 to 55 dynes per cm. The values for human blood were similar. It was found that the surface tension of the plasma of dogs exposed to 450 r total-body X irradiation increased in the postirradiation period, reaching levels of 56 to 60 dynes per cm. In many, this increase was found to parallel the increase in the whole-blood clotting time (Figures 39 and 40), but there were several outstanding exceptions. It will be noted that the whole blood clotting time often showed a terminal return to normal, while the surface tension remained elevated (Figure 39). The increases in plasma surface tension occurred earlier and were still present when changes in the platelet count and protamine titration appeared. In only a few animals did the concentration of total proteins and/or albumin appear to correlate with plasma surface tension; that is, when the protein was low in concentration, the surface tension was high and vice versa (Figure 40).

In an attempt to obtain some idea of the order of magnitude of changes produced in the surface tension by changes in protein concentration, the following experiment was performed. Solutions of bovine albumin (Armour Fraction IV) in normal saline were made in concentrations varying from 2 g per cent to 4 g per cent, and the surface tension of each solution was measured (Table 22). The surface tension of 4 g per cent solution of albumin in normal saline was 53.9 dynes per cm, a value comparable to that of dog plasma. As the concentration of albumin was lowered, the surface tension of the solution increased, as was expected, since less surface active material was present at lower concentrations. At an albumin concentration of 2 g per cent, the surface tension was 55.2 dynes per cm, thus a change in albumin concentration of 2 g per cent caused an increase in surface tension of 1.3 dynes per cm. The increases in surface tension seen in the plasma

Figure 39. Increase in plasma surface tension in a dog following 450 r total-body irradiation, and its relation to whole-blood clotting time.

Figure 40. Increase in plasma surface tension in a dog following 450 r total-body X irradiation, and its relation to whole-body clotting time.

of irradiated dogs were greater, ranging from 2 to 6 dynes per cm. These increases are out of proportion to the changes in protein level when compared to the in vitro albumin system as discussed above. Again, it may point to changes in the particulate nature of the plasma protein. It will also be noted from Table 22 that differences in level of albumin of 0.3 g per cent produced no detectable change in surface tension by the method employed.

Table 22

The surface tension of bovine albumin solution of various concentrations

G % Albumin	Surface tension (dynes/cm)
4.0	53.9
3.55	54.0
3.2	54.3
2.9	54.2
2.66	54.4
2.46	54.7
2.28	55.0
2.13	54.2
2.0	55.2

The investigation of the in vivo surface with which blood comes in contact, that is, the intimal lining of blood vessels, was carried out by observing the angle of contact (degree of wetting) of the intimal surface by droplets of various liquids. Segments of aorta and vena cava, 6 to 7 cm in length, were excised from dogs in acute irradiation experiments. These were opened longitudinally and pinned out flat on a cork base plate. The blood remaining in the segment was gently removed by suction, and droplets less than 1 mm in diameter were carefully placed on the intimal surface from finely drawn glass capillaries. The angle of contact between the liquid and the intima could then be observed. Droplets of freshly drawn blood (using no anticoagulant) were found to make an angle of contact of about 80 to 90° with the intima (Figure 41). This is comparable to the angle of contact that fresh whole blood (with no added anticoagulant) makes with a paraffin surface (Figure 37). Blood to which various anticoagulants were added in standard amounts (including oxalate, citrate, and heparin), as well as plasma from these bloods, made similar angles of contact. Droplets of distilled water and of normal saline made angles of contact of 100 to 110° with intimal surfaces. These findings were the same for both arterial and venous segments. Thus, the surface of normal intima is somewhat hydrophobic; is wet to a small degree by water, whole blood, and plasma;

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Figure 41. Angle of contact of whole blood against normal vascular intima.

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and therefore, should act as an in vivo factor in aiding the maintenance of the fluidity of circulating blood. Intimal surfaces that were scraped lightly with a scalpel blade were found to be more wettable by blood than normal intima, a smaller angle of contact being made (Figure 42). The importance of injury to vascular intima in relation to intravascular thrombosis has long been recognized. Our results graphically demonstrated one of the mechanisms involved in this condition. In addition, this simple experimental tool offers possibilities for investigation of vascular lining in various inflammatory and degenerative diseases.

Whole blood and plasma viscosity: The viscosity of whole blood was found to be largely dependent upon the concentration of the erythrocytes. Presumably the platelet and leucocyte levels also contribute to the values obtained, but these were not directly investigated, although in irradiated animals transfused to plethoric levels, the viscosity greatly increased at a time when the concentrations of platelets and leucocytes were very low.

The plasma viscosity changes, while definite, were not as marked as those of whole blood and are not strictly comparable.

In general, the plasma viscosity was somewhat increased (Figure 43). This did not appear to be a function of plasma protein concentration which, if showing any change, usually decreased until the terminal phase. These findings suggest the possibility of some change in the physical state of the plasma proteins rather than in concentration.

DISCUSSION

It is known that colloid particles in a hydrated state will cause an increase in viscosity as well as surface tension, so that either ionization or molecular aggregation of plasma proteins should cause an increase in plasma viscosity since these particles are more hydrated than proteins in a molecular state.⁽¹⁾ Thus, using the viscosity of plasma relative to water as an indicator, it was shown that though the plasma viscosity in part depends on the level of proteins (Figure 43), in some cases the viscosity increase is independent of change in protein levels, so that one must postulate some other mechanism for the increased viscosity in these instances.

In this connection, the observations of the in vitro effects of X rays on serum and egg white by Nakashima,⁽²⁾ and by Wels and Thiele,⁽³⁾ are of interest. They observed precipitation of these proteins by X rays. Clark⁽⁴⁾ found that X rays produced coagulation of colloidal clay slips as measured by increases in viscosity. He found this to be a function of the amount of organic protein material present.

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Figure 42. Angle of contact of whole blood against scraped vascular intima.

Figure 43. Plasma viscosity in this dog does not correlate with levels of plasma proteins.

The physicochemical factors that have been discussed probably play a role in the delicate balance between the fluid-solid states of mammalian blood.

These factors, acting in summation with chemical, enzymatic, and immunologic forces probably contribute to the system in either a fluid or solid state. If any one of these factors is altered, the equilibrium may be shifted toward increased or decreased coagulability. If two or more of these factors are altered even to a minute degree, the summation of these changes may be powerful enough to influence the equilibrium. It is probable that the influence of any one of these factors is different under different circumstances of clot formation. Therefore, in order to understand pathological coagulation, whether it be increased or decreased coagulability, one must investigate all of the component forces known.

The greatest limitations in studies of this nature are the methods presently available. They are gross and lack the preciseness necessary to apply to small amounts of material or to small areas for measurement of changes.

SUMMARY

1. An increase in plasma surface tension has been observed following total-body X irradiation in the dog.
2. This increase can be explained in part on the basis of changes in both the concentration and the state of particulate aggregation of plasma proteins.
3. The in vivo changes are greatly out of proportion to the measurable in vitro changes of surface tension with variations in protein concentration.

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9. FURTHER STUDIES ON THE ISOLATION OF HEPARIN FROM BLOOD OF IRRADIATED DOGS*

by
J. M. Sutherland and J. G. Allen

INTRODUCTION

Further experience with attempts to isolate heparin from the blood of dogs exposed to 450 r total-body X irradiation is detailed in this part.

MATERIALS AND METHODS

Whole-blood clotting times⁽¹⁾ and protamine titrations⁽²⁾ were determined to be normal for a series of mongrel dogs, and the dogs were exsanguinated. One sample of this normal dog blood with no added heparin was extracted for anticoagulant activity. In addition, aqueous beef sodium heparinate (Abbott) was added to other samples in concentrations of 1 mg per cent (790 ml of blood), 10 mg per cent (400 ml of blood), and 20 mg per cent (550 ml of blood), and these specimens of blood were extracted for heparin. Ten mg of heparin were given intravenously to an 8.5-kg dog, the collection of blood was started in 3 min., and this blood (540 ml) was extracted. Clotting times, protamine titration, and platelet counts were also determined for a series of irradiated dogs. These dogs were exsanguinated and their blood was extracted for heparin.

Bleeding technique: Using a glass canula inserted into the femoral artery after visualization under procaine infiltration, or using a 15-gauge needle inserted directly through the skin into the artery, blood was collected into a transfusion bottle until the flow of blood ceased. Sodium citrate was used to keep the blood incoagulable.

Selection of a method of extraction of heparin from blood: The method of extraction presented by Jaques and Waters⁽³⁾ differed from the methods that are used commercially, especially in the purification of the crude product. It was determined that a crystalline barium salt could be prepared from the various commercial sodium salts of heparin and from a specially prepared sodium salt of heparin prepared from dog liver (Abbott). In view of these facts, it was decided to modify the procedure given by Jaques and Waters so that it would be more parallel to the procedures used by the various pharmaceutical houses.

*This work was supported by the Medical Department, U. S. Army, the Biological and Medical Research Division of the Argonne National Laboratory, and the Department of Surgery of the University of Chicago.

Our crude product was precipitated by alcohol after dialysis and tryptic digestion was done in accord with the method of Jaques and Waters. This part of their procedure is a modification for blood of the classical method of Scott and Charles.(4,5,6)

After treatment with ammonium carbonate, the crude product was purified by fractional precipitation with alcohol as described by Kuizenga.(7) This intermediate purification product was dissolved again in ammonium hydroxide, ammonium carbonate, and water; charcoal (3 per cent) was added, the pH was adjusted to 6.0 with acetic acid, and impurities were adsorbed during treatment at 60°C for one hour. After cooling, fractional precipitation was again carried out and the purified product was removed by centrifugation. Any remaining particulate matter was removed after dissolving at pH 8.0 (sodium hydroxide). The product was precipitated with alcohol at pH 6.0.

The barium salt was prepared according to the method of Charles and Scott(8) for this method gave identifiable crystals most consistently. In general, the best crystals and the most active materials were precipitated earlier, at a higher temperature, and with a lower acetic acid concentration.

Bioassay of anticoagulant substances: The assay method of Foster(9) was performed, utilizing two series of tubes, each containing 1 ml of beef plasma. The solution of unknown potency is added in increments to the tubes of one series and a solution of known potency of heparin is added to the tubes of the second series. The volume is equalized with 0.9 per cent sodium chloride solution. Calcium chloride solution is added to the two series of tubes and they are put in a 37°C constant temperature bath for one hour. The end-point is taken as the tube which contains the smallest amount of heparin or unknown solution and which does not clot completely in one hour. This is a valid and reproducible end-point.(10,11)

The unknown solutions were assayed against powdered sodium heparinate (100 units per mg) obtained from Connaught Laboratories. This standard was assayed against the Provisional International Standard.(12) The unit is defined as 1/130 mg of a standard sodium salt of heparin and is approximately equal to the Toronto unit proposed by Murray and Best(13) which is equal to 1/100 mg of a standard barium salt of heparin.

EXPERIMENTAL RESULTS

Results of extraction of normal dog blood with and without added heparin: No anticoagulant activity was found in the extract of normal blood to which no heparin had been added (Table 23). Although neither crystals nor measurable anticoagulant activity were present in the final product of the blood that contained 1 mg per cent of heparin, the barium acetate residue had some anticoagulant activity (1.5 per cent of the activity

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Table 23

Recovery of heparin added to the blood of normal dogs

Blood			Heparin added			Heparin concentra- tion Mg/100 ml	Heparin recovered as barium salt					
Vol ml	Pro- tamine titra- tion μg	Clot- ting time in min.	Ml	Mg	Units		25°C first crop		0°C second crop		Total	
							Units per mg	Mg	Units per mg	Mg	Units	Yield per cent
550	120	25	11.00	110.0	11000	20	89	11.6	70.4	2.0	1240*	11.2*
400	120	25	4.00	40.0	4000	10	20.5	1.3	**		26.6	0.7
790	120	30	0.79	7.9	790	1	**		**			0.0
540***	100	35	1.00	10.0	1000	1	**		**			0.0
1000	120	33	0.00	0.0	0	0	**		**			0.0

*Includes two additional crops in which no crystals were observed 1) after cooling overnight in refrigerator and 2) after addition of 100 per cent more acetic acid and cooling overnight in refrigerator.

**No measurable anticoagulant potency.

***One ml of aqueous heparin was given intravenously and collection of blood was started in 3 min. at which time the clotting time was 300+ min.

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of the added heparin). The barium salt of the product of the blood to which heparin had been added to 10 mg per cent contained characteristic crystals on cooling slowly to room temperature over a period of several hours; however, the product was of low potency and accounted for only 0.7 per cent of the heparin which had been added. An additional 1.5 per cent of the added heparin was accounted for by measurable losses in the charcoal treatment and in the residue formed on addition of barium acetate.

The barium salt of the product of the blood to which heparin was added to 20 mg per cent contained characteristic crystals on slowly cooling to room temperature and additional characteristic crystals on further cooling in an ice bath; these products were 89 units per mg and 70 units per mg, respectively. Further products were obtained after refrigeration overnight and after addition of more acetic acid and cooling overnight. Although crystals could not be identified in these latter products, they possessed measurable anticoagulant potency, 55 units per mg and 12 units per mg. The combined anticoagulant potencies of the four products accounted for 11 per cent of the added heparin. An additional 19 per cent of the added heparin was accounted for by measured losses in the precipitate formed after the addition of ammonium carbonate, in the 30 per cent alcohol precipitate, in the charcoal treatment, and in the residue formed after the addition of barium acetate.

Extraction of the blood of the dog in which heparin was given intravenously yielded no barium salt; 2.8 per cent of the added heparin was lost in the 30 per cent alcohol precipitate. In the extraction of each sample of blood, the major losses occurred in the initial extraction and separation of the protein fraction.

Results of extraction of blood of irradiated dogs: The irradiated dogs, whose blood were extracted, exhibited prolonged clotting times, increased protamine titrations, and decreased platelet counts (Table 24). In addition, not only was there premortem evidence of bleeding from the nose, mouth, rectum, and urethra and into the skin (Figure 44), but also postmortem evidence of macroscopic bleeding into the lymph nodes, gastrointestinal tract, myocardium, and lungs (Figures 45 and 46). Despite the amount of observed bleeding, no extractable anticoagulant was found in the blood of any of these animals; nor was any measurable anticoagulant activity found in any of the discarded precipitates, residues, supernatants, or filtrates that were assayed.

Table 24

Dogs whose blood was extracted for anticoagulant material:
clotting time, protamine titration, platelet count, and
number of days after 450 r of total-body irradiation

Dog number*	Volume of blood ml	Clotting time† day of death min.	Protamine titration† μg	Platelet count 1000/cmm	Days post- irradiation
402	140	240	140	38	10
404	100	42	140	50	12
407	390	185	140	78	8
408	335	60	160	40	12
405	180	300	140	36	13
414	285	120	160	26	11
406	480	51	160	26	15
413	485	180	140	27	11

*Blood obtained from dogs 402, 404, and 407 was pooled and that from 408, 405, and 414 was separately pooled.

†Determinations were made in 24-hr period preceding death.

DISCUSSION

A clotting inhibitor in the blood as the cause of the increase in clotting time was suggested by this laboratory.⁽¹⁴⁾ It was observed that if the clotting time of the blood of an irradiated dog was sufficiently prolonged, it would sometimes, but not always, delay the clotting of the blood of a normal dog. It was thought the substance responsible for inhibition of clotting was similar to but not identical with heparin, for specific anti-heparin substances prevented or diminished hemorrhage in some, but not all, irradiated dogs in vivo and shortened their prolonged clotting time.

The present work has outlined a procedure whereby a crystalline barium salt of heparin can be isolated and identified from blood when the heparin concentration is 10 mg per cent or above. Heparin is not present in these concentrations in the blood of irradiated dogs. However, the amount of heparin necessary to give clotting time changes in irradiated dogs is exceedingly small. It has been calculated that the amount necessary

DOG 406

Figure 44. Dog 406.

Soft tissue hematoma in the neck as seen at autopsy on the 15th day after irradiation (450 r). See Figure 45.

DOG 406 - 450r

Irradiated 1-13-50

Died 1-28-50

Aureo.

Figure 45. Dog 406.

Abdominal viscera as seen at autopsy on the 15th day after irradiation showing hemorrhage in the mesentery of the ileum and at the site of femoral venepunctures. This dog also had epicardial, endocardial, gastric, and rectal petechiae.

Figure 46. Dog 404.

Thoracic viscera as seen at autopsy on the 12th day after irradiation (450 r) showing hemorrhagic pneumonic consolidation of the right lung, epicardial petechiae, and mediastinal hemorrhage. This dog also had hemorrhage in the lung hila, at the site of venepunctures in the femoral regions, and in the mucosa in the region of the larynx and in the trachea. There were petechiae in the serosa of the stomach and in the mucosa of the stomach, duodenum, colon, and gall bladder.

to give the clotting time changes observed in the irradiated, thrombocytopenic dogs (Parts 3 and 4) whose blood was extracted is between 0.1 and 0.7 μg per cent or roughly between $1/6$ and $1/40$ the amount of heparin necessary to give comparable changes in normal blood. Dog heparin is said to be $2\frac{1}{2}$ times as active as beef heparin.⁽¹⁴⁾ The amount of dog heparin necessary to give these changes in thrombocytopenic blood would be 0.0004 to 0.0028 mg per ml or 4 to 28 μg per cent. These quantities are below the amounts detectable by the extraction procedure described in this paper. On the other hand, the amount of heparin necessary to increase the protamine titration 0.02 mg per ml is 0.018 mg per ml or 1.8 mg per cent of heparin. Although a heparin or heparin-like substance may be the cause of prolonged clotting times and the increased protamine titration, in the irradiated dog, it is certainly not a substance of the potency of commercial beef heparin.

In a previous report from this laboratory,⁽¹⁴⁾ the method of Jaques and Waters⁽³⁾ was used to extract heparin from the blood of irradiated dogs (450 r, total-body). Anticoagulant material was obtained in yields of from 0.93 to 2.3 mg per 100 ml of blood. The products had potencies comparable to heparin of 90 to 110 units per mg. These quantities exceeded the amounts of heparin necessary to cause complete incoagulability of the blood, yet the blood in these animals did clot within an hour. Although the method of determining clotting time then was not as sensitive as the one used now, the clotting times in these earlier experiments ranged from 47 to 87 min. with an average of 67 min. The clotting times of the dogs in the present series were at least comparably prolonged. We cannot account for the different results obtained in these two reports; we cannot neglect the fact that save for our first report, all other attempts in this laboratory have failed to give conclusive evidence that heparin is present in increased amounts in the blood after X irradiation.

In this connection, changes reported in mast cells may be of interest. For example, Sylven,⁽¹⁵⁾ as early as 1940, found that following irradiation of the abdominal wall of rats, there was a diminution in the granules of the mast cells and a diffuse metachromatic staining throughout the corium and connective tissue appearing as early as 24 hr after exposure. Campani⁽¹⁶⁾ recently has reported similar observations on the mouse. Sylven observed no tendency for the mast cell granules to reappear during the 17-day survival period. Although the studies of Sylven, Campani, and others are indeed suggestive that "heparin" or one of its analogues may be released into the surrounding tissue after irradiation, there is normally present a polysaccharide in ground substance that stains metachromatically. There has been work to suggest the possibility that other substances as well as heparin may account for the diffuse metachromasia after irradiation.

SUMMARY

1. A method is presented for the extraction of heparin from blood, by which crystals of the barium salt of heparin can be prepared and identified from blood to which heparin has been added in amounts of 10 mg per cent or larger. The amount of heparin necessary to cause prolonged clotting time in irradiated dogs is 4 to 28 μ g. per cent, or less than 5 per cent of the least amount detectable by the method employed.
2. By this procedure, no anticoagulant could be extracted from the blood of irradiated dogs.

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10. IN VIVO CHANGES IN THE ULTRAVIOLET ABSORPTION SPECTRUM OF DOG PLASMA FOLLOWING EXPOSURE TO X IRRADIATION*

by
K. A. Lathrop, R. F. Gilmore, and J. G. Allen

INTRODUCTION

A study was undertaken of the ultraviolet absorption spectrum of plasma of dogs following exposure to 450 r total-body X irradiation. It was hoped that additional information could be obtained on the nature of the damage produced by such ionizing rays, particularly in regard to the clotting defect.

As previously reported,⁽¹⁾ plasma from normal dogs was found to give a characteristic curve in the ultraviolet with the minimum at 251 m μ and the maximum at 278 m μ . These values are in agreement with those reported by Lecomte du Nouy⁽²⁾ for normal mammalian serum or plasma ($\lambda_{\text{max.}} = 2764 \text{ A} \pm 5\text{A}$, and $\lambda_{\text{min.}} = 2495 \text{ A} \pm 5\text{A}$).

The characteristic ultraviolet spectrum of blood plasma is generally believed to be produced by the presence of the three aromatic amino acids, phenylalanine, tyrosine, and tryptophan.^(3,4,5) In most proteins absorption by phenylalanine can be disregarded because of its low molecular extinction.

Tryptophan shows these absorption characteristics, $\lambda_{\text{min.}} = 244 \text{ m}\mu$, $\lambda_{\text{max.}} = 278 \text{ m}\mu$, $E = 5.43 \times 10^{-3}$; and for tyrosine (pH = <8), $\lambda_{\text{min.}} = 245 \text{ m}\mu$, $\lambda_{\text{max.}} = 275 \text{ m}\mu$, $E = 1.54 \times 10^{-3}$. Marenzi and Villalonga^(6,7) observed that the aliphatic amino acids absorb ultraviolet light in an approximately straight line relationship before reaching total extinction between 240 and 250 m μ . The $\lambda_{\text{min.}}$ of 251 m μ for plasma can be accounted for by this additional absorption of the aliphatic acid constituents.

The ultraviolet absorption characteristics of the various amino acids, indole and benzene compounds, and amino acid mixtures have been determined in this laboratory under the same conditions as the plasma analyses. Our data are in good agreement with those of the above-mentioned investigators.

*This work was supported by the Biological and Medical Research Division of the Argonne National Laboratory, the Medical Department, U. S. Army, and the Department of Surgery of the University of Chicago.

EXPERIMENTAL

Determinations of ultraviolet absorption spectra were made with the Beckman quartz spectrophotometer, model DU, using the hydrogen discharge tube and silica cells, 1.00 cm in thickness. Temperature variation of the samples, while determining the curve, usually was from 25° to 28°C.

Plasma was prepared by centrifuging 3 to 4 ml of oxalated arterial blood (drawn into an oiled syringe, then transferred to a tube containing 0.0184 g of potassium oxalate). This plasma was diluted in the ratio of 1:100 with normal NaCl solution. The resulting solutions were about pH 6.5. Blanks were normal NaCl solution. Beginning at 235 m μ (total extinction at lower wave-lengths) readings were made at intervals of 5 m μ , except in the region of the maximum and minimum where they were made more frequently. During the postirradiation period, daily determinations were made.

The extinction values were plotted on 20 x 20 per inch coordinate paper. Extinction percentages were not converted to extinction coefficients, since, by the Beer-Lambert equation

$$E = \frac{\log \frac{I_0}{I}}{cd} \quad (1)$$

When c and d are constant, $E = \log 100 - \log I$, $= 2 - \log I$ (2) and, $E\% = 100 - I\%$ (3). Therefore equations (2) and (3) are proportional.

OBSERVATIONS

The ultraviolet absorption spectra of plasma from about 25 dogs has been observed during the pre- and postirradiation periods. In general, the trend presented by the plasma of each animal was similar. Data obtained from a representative animal, dog 4, is graphically presented in Figure 47.

At no time after irradiation has a shift been observed in either the maximum or minimum wavelength. The λ_{max} , consistently falls at 278 m μ and the λ_{min} , at 251 m μ .

There was an initial decrease in the total extinction by the plasma. A minimal extinction value was reached 4 to 6 days following irradiation,

Figure 47. Ultraviolet absorption spectra of plasma of a representative dog.

after which there was a return toward the pre-irradiation value. Two or 3 days prior to death, the pre-irradiation extinction value was exceeded and a maximum value was reached either on the day preceding or the day of death.

The decreases in extinction following irradiation appear to parallel the pre-irradiation absorption values, and thus to indicate a decrease in the plasma of proteins containing the aromatic amino acids. Plasma proteins as determined by the Kjeldahl nitrogen method also decrease following irradiation, reaching a minimum value between the fourth and sixth days.

Likewise, the increased extinction values of the last few days of life appear to parallel the pre-irradiation values. A few plasmas showed greater increases at 250 to 260 $m\mu$ and at 285 to 295 $m\mu$ than in other portions of the curve.

DISCUSSION

2/ Simultaneous plasma protein and albumin-globulin assays have been made on a large number of the animals studied. The ultraviolet absorption at 278 $m\mu$ closely parallels the protein level, irrespective of quantitative changes in the albumins and globulins. 3/ This may mean 1) that the albumins and globulins of dog plasma, as determined by precipitation with 22 per cent Na_2SO_3 , are nearly equal in tryptophan and tyrosine content; 2) that no increase of free tryptophan or tyrosine occurs in the plasma following irradiation. An increase of either of these acids, or any of their metabolic products containing the ring structure, would increase the absorption above the protein level. If present investigations prove these two things to be true, quantitative measurement of dog plasma proteins by absorption of ultraviolet light can be made more readily and accurately than by Kjeldahl nitrogen determination.

Careful analysis of the curves may reveal consistencies in changes which do not parallel the normal plasma curve (such as those already mentioned at 250 to 260 $m\mu$ and 285 to 295 $m\mu$). If this is found true in a large number of cases, it may indicate the presence of additional compounds, or an increase in compounds normally present which have a low extinction. Other investigators⁽⁴⁾ have believed that there is no evidence for the presence of light absorbing groups other than the three aromatic amino acids. However, irradiation may change the normal conditions. Experiments are in progress to observe the effect of in vitro irradiation on the aromatic amino acids and normal dog plasma.

3/ The data must undergo more critical analysis before more definite conclusions can be made concerning the significance of the observations. 3/ Subsequent papers will report this work in greater detail. ~~END~~

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11. CORRELATED STUDIES ON THE IRRADIATED DOG*

by

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INTRODUCTION

A number of blood and plasma abnormalities in the irradiated dog have been presented in previous sections. In an attempt to correlate these abnormalities, three dogs were given 450 r total-body X irradiation and the following studies were made 1) complete hemocytology; 2) coagulation survey; 3) plasma proteins; 4) surface tension and viscosity measurements; 5) plasma ultraviolet light absorption; and 6) protein and proteose sulfhydryl polarographic curves.

The methods, with the exception of the polarographic data, have been presented in detail in the preceding papers. The polarographic techniques of Mueller and Davis^(1,2) were employed.

EXPERIMENTAL

Dog 93 (Figures 48 to 52) lived only 6 days after the exposure; death occurred before the thrombocyte level had decreased below 190,000. Leucocytes were almost absent by the third day suggesting that an irradiation dosage above 450 r had been given.

In this short period of time, the whole-blood clotting time showed a prolongation, the protamine titration increased, and there was a rise in plasma antithrombin titer.

Plasma protein studies were inadequate for presentation.

Physical measurements showed little change in plasma or whole-blood viscosity (only a slight anemia was seen), but the surface tension was markedly increased.

Ultraviolet light absorption studies on plasma showed the characteristic early decrease and terminal rise in extinction between 251 and 278 m μ .

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DOG 93 IRRADIATED 4-8-49 DIED 4-15-49

Figure 48. Platelet, red blood cell, and white blood cell values of dog following exposure to 450 r total-body X radiation.

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Figure 49. Coagulation survey of dog following exposure to 450 r total-body X radiation.

DOG 93 IRRADIATED 4-8-49. DIED 4-15-49

Figure 50. Surface tension and viscosity of blood of dog following exposure to 450 r X radiation.

Figure 51. Ultraviolet light absorption of plasma of dog following exposure to 450 r total-body X radiation.

Figure 52. Polarographic protein waves showing a decrease in protein sulfhydryls and an increase in the proteose sulfhydryls following exposure to 450 r total-body X radiation.

The cobalt catalyzed sulfhydryl protein waves indicated a decrease in the protein sulfhydryl (digest wave) and an increase in the small molecule or proteose sulfhydryls (filtrate wave).

Dog 757 (Figures 53 to 58) lived 12 days after X irradiation. Hemocytologic studies were representative of most of the animals similarly irradiated, demonstrating a rapid fall in the leucocytes, a later but precipitous fall in the platelets, and a progressive anemia.

Clotting times were irregular but markedly increased in the late postirradiation period. There was a terminal rise in the protamine titration and a mild decrease in prothrombin activity.

Plasma protein changes followed no regular pattern, but there was a slight decrease in plasma albumin concentration with only a slight rise in the globulins.

There was a rise in plasma surface tension. On the 10th post-irradiation day, there was a marked fall associated with a normal clotting time. The whole blood viscosity decreased as the anemia progressed. There was a rise in the plasma viscosity, although there were no marked quantitative protein changes.

The ultraviolet light absorption maxima and minima were characteristic with an early decrease and a late rise; the latter values were greater than those of the pre-irradiation period.

The digest protein sulfhydryls remained relatively unchanged, but there was a sharp increase in the smaller molecule sulfhydryl compounds.

Dog 112 (Figures 59 to 64) lived 9 days after irradiation, and the changes in the cellular elements of the blood were again characteristic of the dog in the postirradiation period. Changes in the clotting mechanism were also characteristic, showing increases in the whole-blood clotting time, antithrombin level, and protamine requirement. Prothrombin levels were normal.

The physical changes were similar to those seen in the other animals reported.

Ultraviolet light absorption curves showed a fall from the original values and a terminal rise which, however, was short of the original level.

Polarographic curves presented an unusual fall in the protein sulfhydryls; however, the proteose sulfhydryl values showed a rise during the postirradiation period.

Figure 53. Platelet, red blood cell, and white blood cell values of dog following exposure to 450 r total-body X radiation.

Figure 54. Coagulation survey of dog following exposure to 450 r total-body X radiation.

Figure 55. Plasma protein changes in dog following exposure to 450 r total-body X radiation.

Figure 56. Surface tension and viscosity of blood of dog following exposure to 450 r total-body X radiation.

Figure 57. Ultraviolet light absorption of plasma of dog following exposure to 450 r total-body X radiation.

Figure 58. Polarographic protein waves showing an increase in proteose sulfhydryl with protein sulfhydryl essentially unchanged.

Figure 59. Platelet, red blood cell, and white blood cell values of dog following exposure to 450 r total-body X radiation.

Figure 60. Coagulation survey following exposure to 450 r total-body X radiation.

Figure 61. Plasma protein changes in dog following exposure to 450 r total-body X radiation.

Figure 62. Viscosity and surface of plasma following exposure to 450 r total-body X radiation.

Figure 63. Ultraviolet absorption spectra of plasma of dog following exposure to 450 r total-body X radiation.

Figure 64. Polarographic protein waves following exposure to 450 r total-body X radiation.

COMMENT

These data were collected from three animals, and large quantities of blood had to be drawn each day. The results, however, correspond with the findings in other irradiated dogs in which the effect of exposure upon each of these analyses was made.

One of the reasons why these studies were made was the hope that some of these functions might serve as an index of the severity of X-ray exposure. No group of changes could be demonstrated shortly after irradiation that could be used to indicate the lethality of the dose.

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12. SUMMARY OF IRRADIATION EFFECTS ON THE BLOOD COAGULATION MECHANISM*

by

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M. H. Sanderson

INTRODUCTION

The hemostatic mechanism is more complicated than can currently be explained, and the damages produced by total-body X irradiation are so far reaching that most, if not all, of the clotting mechanism may be directly or indirectly affected. In addition, there is evidence of widespread vascular damage about which little is known. Since the normal hemostatic mechanism is not well understood and the effects of X irradiation are only partly known, rational and complete treatment of irradiation hemorrhage cannot yet be established. However, certain features of the pathogenesis of this syndrome are established, and limited therapeutic aid is available.

A summary discussion on certain of the phenomena described in the previous parts is presented in this section. Some recent data are included to augment the discussion.

THE WHOLE-BLOOD CLOTTING TIME

In many instances, the clotting times were prolonged during the latter half of the postirradiation period in our animals. In some animals this was more pronounced than in others. Incoagulable bloods were rarely found, being observed only three times in the course of 665 clotting time determinations of the 79 animals whose data are presented in Part 2 of this series. Once the clotting time increased, it did not necessarily remain abnormal until death as normal values were sporadically observed, and particularly during the terminal state. In addition, five animals revealed no detectable increase in clotting time during the postirradiation period.

During the last forty years, several investigators⁽¹⁻⁶⁾ have reported a delay in coagulation following total-body X irradiation of both man and the experimental animal. However, some^(7,8) have failed to detect any clotting time change. These differences in experience may in part be due to the fact that the clotting times are not usually greatly

*This work was supported by the Medical Department, U. S. Army, the Biological and Medical Research Division of the Argonne National Laboratory, and the Department of Surgery of the University of Chicago.

prolonged in the irradiated dog and that clotting time increases of these magnitudes can be easily obscured by the usual techniques as illustrated in Part 1. In fact, no method yet devised as a measure of the in vitro clotting activity of blood can be construed to simulate completely in vivo coagulation or to be free of accelerating artifacts. It is our belief that the clotting time is of value only when artifacts are reduced to a minimum, and that under these conditions some abnormalities are revealed that otherwise would not be recognized. Because all animals do not develop an increase in the clotting time, a small series of animals may give misleading information.

A CLOTTING INHIBITOR

Anticoagulant effect on normal blood: In the blood of some of our irradiated dogs, an inhibitor was demonstrated. When present, it was generally weak and difficult to detect. A striking exception to this experience was dog 108, described in a previous report.⁽⁹⁾ The blood of this animal could not be made to clot even when beef lung thromboplastin was added. Since four samples of blood drawn over a 48-hr period all showed the same phenomenon, it seemed unlikely that the results were artifacts. Furthermore, the blood and plasma of this animal delayed coagulation of normal dog blood and plasma. This clotting inhibitor was overcome in vitro on addition of toluidine blue, and neither the increased clotting time nor the clotting inhibitor could be detected after the dye was administered intravenously.

In the animals subsequently studied, the bloods of some were tested for anticoagulant activity when the clotting time was greatly prolonged, and since this was uncommon, only a few were examined. Moreover, some of these animals were given either toluidine blue or protamine sulfate thus ruling out the possibility of such studies. Of ten animals in this series examined for a mixed whole-blood clotting time, three showed evidence of mild anticoagulant activity (Table 25). Furthermore, when these bloods were diluted with increasing increments of normal saline, the clotting times were shortened in low dilutions, but as the amounts of saline were increased, the clotting times were prolonged (Figures 65 and 66 and Table 26). In three animals, we were unable to detect the presence of an inhibitor by either method even though the clotting times were prolonged 3 to 4 times the normal value. The frequency with which a clotting inhibitor accounts for the prolonged clotting time is not known, although of 14 dogs tested by mixed plasma (recalcified), mixed whole blood and/or saline dilution techniques 7 dogs' bloods have shown anti-coagulant activity.

Is the inhibitor heparin?: As described in Part 9 beef heparin added to dog blood has been extracted and crystallized. Ten animals were exsanguinated late in the postirradiation period when the clotting times were

Table 25

Mixed whole-blood clotting time results in X-irradiated dogs

Dog	Day*	C.T.**	P.T.***	Per cent normal blood added												
				0	10	20	30	40	50	60	70	80	90	100		
342	16	150	>150	75'	110'		46'		42'	46'	29'	27'	21'	20'		
402	10	240+	220	40'	75'	52'	45'	40'	46'	40'	35'	18'	20'	16'		
424	7	165	120		75'	70'	65'	51'	47'	44'	35'	35'	30'	20'		
405	13	>300	140	45'	7'	7'	7'	8'	13'	8'	11'	24'	18'	8'		
406	15	51	>160	15'	8'	9'	9'	10'	10'	10'	15'	10'	10'	8'		
408	12	36	150	14'	9'	9'	9'	12'	9'	9'	9'	9'	9'	9'		
413	11	180+	140	19'	13'	19'	11'	11'	11'	10'	10'	10'	11'	10'		
414	11	120	>160	12'	15'	15'	15'	15'		15'	15'	12'	12'	15'		
429	12	310	120	16'	24'	24'	24'	23'	16'	16'	16'	7'	12'	14'		
431	12	275	140	15'	11'	11'	15'	15'	15'	15'	15'	15'	15'			

*Number of days postirradiation 450 r.

**Whole-blood clotting time in minutes.

***Protamine titration in μg .

Figure 65. Effect of saline on whole-blood clotting time of dog.

Figure 66. Effect of varying amounts of saline on whole-blood clotting time of patients.

Table 26

Serial saline dilution clotting time results in X-irradiated dogs

Dog	Day*	C.T.**	P.T.***	Added saline in ml								
				0.1	0.25	0.5	1.0	2.5	3.75	5.0	7.0	0.0
405	13	> 5 hrs	140	40	26	19	26	60	102	>180	>180	21
429	12	310	120	47	40	30	38	65	84			43
431	12	275	140	20	20	35	40	53	86			55
434	7	165	120	39	70	65	58	70				155
402	10	4 hrs+	220	7	7	7	7	16	29	36		7
406	15	51	>160	19	23	24	24	42	> 90	> 90	> 90	21
408	12	36	150	12		12	12	54				12
413	11	3 hrs+	140	19	24	19	22	24	>140	>140	>140	19
414	11	120	>160	18	18	20	42	42	> 60	> 60	> 60	15
436	6	40	100	14	18	20	37	50	62			15

*Number of days postirradiation 450 r.

**Whole-blood clotting time.

***Protamine titration in μ g.

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moderately prolonged or the protamine titration markedly increased, and the specimens were then extracted for heparin. In these animals, no evidence of either anticoagulant or crystalline material resembling heparin was obtained at any stage of the extraction procedure. These data are in contrast to a previous report from this laboratory in which an anticoagulant material was found to be present at the stage of the sodium salt of heparin. In those extractions, a crystalline heparin preparation could not be obtained, so that the exact identity of the material accounting for anticoagulant activity could not be established. A review of these early data in light of subsequent work suggests that if all the material isolated were heparin, the bloods of the earlier animals should have been incoagulable at the time of exsanguination. Since this was not the case, either an increase of potency occurred during the process of purification or contaminants were present in the final product.

For the present, we believe it is not possible to conclude whether small amounts of heparin reach the blood stream after total-body irradiation and thereby account for some of the clotting changes observed. If heparin is present, it is present only in minute amounts. In severe thrombocytopenia, amounts of the magnitude of $1/15$ th to $1/30$ th of the least effective concentration for normal dog blood can prolong coagulation. Moreover, since the sensitivity of blood to heparin is substantially affected by the platelet concentration and other thromboplastic substances, it is also possible that any heparin bound in this manner could now exist in a free or unbuffered state and exert a retarding influence on coagulation when severe thrombocytopenia obtains. As these studies have progressed over the past six years, it becomes increasingly evident that the mechanism are complex and multiple, and that a significant disturbance in one animal may be absent or insignificant in another.

The protamine titration: This procedure is the measure of protamine sulfate required to clot a standard volume of blood heparinized by a known amount of beef heparin.⁽¹¹⁾ In the normal animal, the protamine requirement is constant for the quantities and potencies of the protamine sulfate and heparin employed. This procedure was designed originally to measure the effects of added heparin in blood; it was hoped that it would also reflect fluctuations of native blood heparin, should such exist. Although this titration can be used to measure or assay heparin in blood, in certain conditions the protamine requirement is inordinately increased while the clotting time remains normal or only slightly increased. If this increased protamine requirement is due to an increase of native heparin of the potency of commercial beef heparin, the blood should not clot. Since increases in the protamine titration were seen fairly frequently and incoagulable bloods were rarely observed, it soon became evident that the titration changes could not be explained by the release of heparin alone, if indeed by this mechanism at all, Figure 67. The blood of a normal dog given enough beef heparin to increase the protamine requirement 0.020 mg is invariably

WHOLE BLOOD CLOTTING TIME AND PROTAMINE TITRATION IN A DOG
FOLLOWING 450r TOTAL BODY IRRADIATION.

A.

CHANGES IN WHOLE BLOOD CLOTTING TIME AND PROTAMINE TITRATION
IN A NORMAL DOG FOLLOWING INJECTION OF VARYING AMOUNTS
OF HEPARIN. BLOOD SAMPLES 10 MINUTES AFTER HEPARIN INJECTION.

B.

Figure 67. A. Whole-blood clotting time and protamine titration in a dog following 450 r total-body irradiation.

B. Changes in whole-blood clotting time and protamine titration in a normal dog following injection of varying amounts of heparin. Blood samples were drawn 10 minutes after heparin injection.

rendered incoagulable. The blood of some irradiated dogs developed increases in the protamine titration of 0.020 mg to 0.080 mg. This was also true of some patients with bleeding abnormalities that seemed to respond to toluidine blue. The increases in the protamine titration in some of these dogs and patients were even more pronounced in some of these which displayed no increase in the clotting time.

In seeking an explanation of this phenomenon, several possibilities were investigated: 1) that the blood contained a heparin-like substance capable of binding protamine, but that this substance had little if any anticoagulant potency; 2) that the material responsible was actually heparin but that its anticoagulant power was reduced because of a deficiency in the plasma co-factor essential for heparin's anticoagulant action; 3) that the increased protamine binding power was due to the liberation of nonheparinoid substances with little or no anticoagulant effect but having the capacity to bind protamine, e.g., nucleic acid substances; 4) that there was no increase in endogenous heparin, heparinoid, or nonheparinoid substances, but that the heparin-protamine complex is more dissociated under certain conditions for reasons unknown at present. These possibilities have been examined as follows:

1. Heparin or heparinoid substances of low anticoagulant potencies and the protamine titration.

When commercial beef heparin was modified by hydrolysis with 11 per cent acetic acid or by blocking the free amino groups with 37 per cent formalin, the anticoagulant potency was reduced. These modified heparins however, also required less protamine to neutralize the residual anticoagulant effect (Tables 27 to 30).

Sodium polyanethol sulfonate (Liquoid-Hoffman-LaRoche), a synthetic heparin-like preparation, and germanin (suramin), a sulphuric acid aromatic compound also containing at least one free amino acid group, were also studied, (Table 31). These substances had extremely low anticoagulant potencies, the effect appearing in concentrations close to amounts which altered the physical nature of the blood. The anticoagulant activity was reversed by protamine sulfate although the mg for mg requirement was much lower than that of heparin. When used in addition to heparin in a protamine titration, this weight relationship was fairly well maintained.

2. Reduced plasma heparin co-factor and the protamine titration.

This possibility appears remote for two reasons: 1) the heparin sensitivity of blood of irradiated thrombocytopenic dogs is greatly increased rather than diminished as would be anticipated with co-factor deficiency; and 2) the addition of normal blood that contains co-factor decreases rather than increases the clotting time of the blood of the irradiated animal.

Table 27

Anticoagulant action of acetic acid hydrolyzed heparin

Approx. mg heparin per ml blood		2	4	6	8	10	12	14	16	18	20	22
Abbott Heparin	1 hr	C	C	C	F	F	F					
	2 hr		C		C	C	F					
B ₁	1 hr		C	C	C	C	C	F	F	F		
	2 hr							C	C	C		
B ₂	1 hr				(F)	F	(F)	(F)	F	F	F	
	2 hr				C	C	C	C	C	C	C	
B ₃	1 hr			C	C	C	C	C	C	C	C	C
	2 hr											

B₁ - 5 ml heparin)
2.5 ml 11% HAc) 24 hr at 68-70°C.

B₂ - 5 ml heparin)
1 ml 11% HAc) 24 hr at 68-70°C.

B₃ - 5 ml heparin)
1 ml 11% HAc) 48 hr at 68-70°C.

C - Solid clot

(F) - Some clotting

F - Fluid

Table 28

Protamine titrations of acetic acid hydrolyzed heparin

Preparation*	Heparin mixture in ml	Protamine titration**
Heparin	0.1	120
B ₁	0.1	60
B ₂	0.1	80
B ₃	0.1	Clotting before titration
B ₃	0.2	80
Heparin)	0.1)	200
B ₃)	0.1)	

*See footnotes of Table 27 re: B₁, B₂, B₃

**Anticoagulants were pipetted into graduated conical tubes in amounts indicated (column 2), then freshly drawn normal dog blood was added to raise the level to 11 ml and thorough mixing was performed. To graded amounts of protamine (0.010 mg increments) one ml of blood was added and mixed. Readings were performed at one hour. The end-point is the smallest amount of protamine at which clotting occurred.

Table 29

Anticoagulant action of formalinized heparin

Heparin mixture in ml*	0	4	6	8	10	12	14	16	20	0
	Clotting time minutes									
D	5	-	70	80	90	-	105	150	160	5
E	5	-	50	65	70	-	120	160	210	5
F	5	55	70	150	180	-	360	-	-	5
G	5	70	70	100	165	390	-	-	-	5
Heparin in μ g	0	4	6	8	10	12	-	-	-	0
Heparin	5	70	195	8 hr	8 +hr	Incoag- ulable				5

D (1.0 ml heparin)
 (0.1 ml 37% formalin)
 (0.1 ml 0.9% NaCl)

E (1.0 ml heparin)
 (0.2 ml 37% formalin) 4 days at 10-15°C

F (1.0 ml heparin)
 (0.2 ml 0.9% NaCl)

G (1.0 ml heparin)
 (0.2 ml 0.9% NaCl) Fresh

*Each ml of D through G heparin mixture contains 8.3 mg of heparin.
 Clotting times were performed on one ml blood.

Table 30

Protamine titrations of formalinized heparin

Preparation*	Heparin mixture in ml	Protamine titration	<u>Protamine</u> <u>Heparin</u>
Heparin	0.1	140	1.54
D	0.1	60	0.8
E	0.1	80	1.1
E	0.2	160	1.1
E	0.3	280	1.23
F	0.1	100	1.32
G	0.1	120	1.59

*See footnote of Table 29 for description of D through G.

Table 31

Protamine titrations of heparin, liquoid, and germanin

Anticoagulants	Mg	Protamine titration	Protamine equivalence of 1 mg in 11 ml
Heparin	1	110	110
Liquoid	2	clots*	-
"	3	"	-
"	6	220	40
"	8	gelatinous change	-
Germanin	6	clots	-
"	8	"	-
"	12	"	-
"	20	600	30
Heparin and liquoid	1 1	140-160	(L) 30-50
"	1 1	150	(L) 40
"	1 2	170	(L) 30
"	1 3	gelatinous change	-
"	1 4	"	-
Heparin and germanin	1 1	130	(G) 20
"	1 2	160	(G) 25
"	1 3	210	(G) 33
"	1 4	250	(G) 35

*Clotting of varying degree in the original blood mixture prevented performing the titration.

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Transfusion of normal blood occasionally prolonged the in vivo clotting time of an irradiated animal, but as far as we know, this has been accompanied by transfusion reactions, (Figure 68).

3. Nonheparinoid materials and the protamine titration.

Protamine and protamine-like substances are said to be present in nuclear material and to be bound to the nucleic acids.⁽¹²⁾ A release of nucleic acids into the circulation as a result of cellular damage from irradiation could account for the increased protamine titration. However, the maximum change in light absorption that might be due to an increase in blood nucleic acid is of the order of 0.04 mg per ml. This is approximately 1/10,000 the amount of thymo or desoxyribose nucleic acid required to produce the minimal effect on the clotting time observed with in vitro studies. Perhaps other possible substances of similar nature exist but since none is yet known, this mechanism seems unlikely.

4. Heparin-protamine dissociation and the protamine titration.

Twenty years ago Fischer⁽¹³⁾ demonstrated that the salts of heparin are governed by the mass law. As such, these salts are subject to the influence of certain ions having the capacity to increase dissociation of the heparin-protamine complex. Data referable to these phenomena are presented in Table 32. These data represent the effect of a salt more than a single ion, although they are well dissociated in solution. It can be seen that the only changes observed were decreases in the protamine titration; so that, within the limits of this type of experiment, there is no evidence of increased dissociation of the heparin-protamine complex.

THROMBOCYTOPENIA

Severe platelet depression invariably occurred in all our animals that survived more than one week after exposure to 450 r. Platelets contribute to thrombi formation in two important ways and perhaps by other mechanisms not yet understood. These are platelet agglutination with adherence at the site of intravascular clotting within the circulation and the liberation of thromboplastin by thrombocytolysis. In man, the fact is well known that thrombocytopenia can exist without abnormal bleeding save for a prolonged bleeding time. Also, we have shown elsewhere,⁽¹⁴⁾ abnormal bleeding corrected by toluidine blue does not directly influence the platelet count, and that the bleeding may be temporarily controlled even though the low platelet count persists.

We are unaware of any experimental means for the production of thrombocytopenia that depresses the platelet count -- doing that and nothing more. As indicated in the preceding parts, the effects of ionizing radiation

Figure 68. Effect of transfusions on the whole-blood clotting time following X irradiation.

Table 32

Effect of certain salts on the protamine titration

Salt	Mg/ml of blood	Protamine titration*
None	-	95
NaCl	0.15	80
	0.75	90
	2.0	80
Na ₂ SO ₄	0.15	95
	0.75	95
	2.0	95
Na ₂ S ₂ O ₄	0.15	90
	0.75	80
	2.0	90
Na ₂ HPO ₄	0.15	90
	0.75	80
	2.0	95
NaH ₂ PO ₄	0.15	90
	0.75	90
	2.0	90
Ca (Lactate)	0.15	90
	0.75	80
	2.0	90

*Solutions were brought to equal volumes with normal saline and approximately 77 μ g heparin per ml blood was present.

are widespread and, in the case of the hemostatic mechanism, many if not all of the components are involved. While in one animal one defect may be more in evidence than another, thrombocytopenia developed in all animals and to the same extreme if the animal survived two weeks or longer. Clinical evidence of bleeding, on the other hand, varied widely and in some animals, gross bleeding was not evident. What then is the role of thrombocytopenia in the pathogenesis of hemorrhage? This question cannot yet be fully answered although there are many ways by which thrombocytopenia can directly account for bleeding or contribute to the continuation of hemorrhage once bleeding has commenced. Where lesions exist, bleeding may occur because the structure of the platelet thrombus is inadequate or because that which does form is not well anchored by platelet adherence to the vessel wall. So far as known, these functions of the platelets are not taken over by any other hemostatic component in thrombocytopenia. Superficial ulcerations of the alimentary tract were frequent complications of irradiation in our animals that bled. In our opinion, this type of bleeding is greatly influenced by the platelet count. Why these lesions occur is not known. Of the possible causes, desquamation, infection, and submucosal punctate hematomata appear prominent. These may act separately or in combination.

To what extent, if any thrombocytopenia contributes to prolongation of the clotting time is problematical. No one has devised an in vitro method by which normal whole blood can be rendered thrombocytopenic without the aid of anticoagulants.

Bearing on the clotting time problem is the fact that within certain limits, platelets inhibit or neutralize heparin. Normal blood can withstand the effects of 20 to 30 times the amount of heparin tolerated by thrombocytopenic blood without increasing the clotting time. More active thromboplastin material can buffer even larger quantities of heparin, Table 33. There is some evidence that heparin is a normal constituent of blood;⁽¹⁵⁾ and, if this is true, it is possible that this quantity of heparin normally is inactivated by the normal platelet count, but in thrombocytopenia, it would be free to influence the clotting mechanism. This would still not explain the presence of abnormal clotting times when platelet levels are normal or near normal or vice versa, (Table 34). Resolution of these possibilities must await the development of better techniques of investigation and more information.

Table 33

Effect of thromboplastin on the protamine titration

Thrombo- plastin in ml	Protamine titration - μ g						
	Time of reading after adding thromboplastin						
	10'	15'	20'	25'	30'	45-240'	24 hr
0	120	110	110	110	110	110	100
0.1	100	80	80	80	80	80	80
0.5	80	70	70	70	60	60	60
1.0	80	60	60	50	50	50	50
2.0	20	15	15	10	10	10	10
4.0	20	15	10	5	5	< 5	< 5

The thromboplastin solution was added to the 11 ml of heparinized blood, mixed, and pipetted immediately.

Thromboplastin (Maltine) solution was made up in a potency that clotted oxalated plasma after recalcification in 12 sec. (1 part plasma, 1 part thromboplastin solution, 1 part calcium chloride solution).

Table 34

Clotting time and platelets
Total-body X irradiation 450 r
Data from 79 dogs

Platelets	Clotting time	No. of occurrences
> 200,000	> 50 min.	8
> 150,000	> 50 min.	3
> 100,000	> 50 min.	18
< 50,000	< 40 min.	43

CAPILLARY INJURY

Disturbances in coagulation do not explain spontaneous bleeding, and it becomes necessary to postulate that diapedesis proceeds to hemorrhagic extent through faulty coagulation or that the vessel wall is primarily disturbed giving rise to spontaneous hemorrhage. Thus far, our studies have not included the vascular bed which is probably one of the most important of the hemostatic disturbances contributing to irradiation hemorrhage.

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