seen to occur. Rhythmic changes in the regional blood flow were observed which were synchronous with but opposite in direction to the changes in systemic blood pressure. This agrees with the conventional view that changes in blood pressure in this situation are secondary to changes in peripheral resistance.

1. Traube, L., *Centralbl. f. d. Med. Wiss.,* 1865, v56, 881.
2. Hering, E., *Sitzber. d. Kaiserl. Akad. Wiss., Wien.,* 1870, v60, 829.
3. Mayer, S., *ibid.,* 1876, v74, 281.
4. Wilson, H. C., *Am. J. Physiol.,* 1936, v116, 295.
5. Irving, L., *Am. J. M. Sci.,* 1938, v196, 988.
6. Andersson, B., Kenny, R. A., Neil, E., *Acta Physiol. Scand.,* 1950, v20, 203.
7. Guyton, A. C., Harris, J. W., *Am. J. Physiol.,* 1951, v165, 158.
8. Burton, A. C., *ibid.,* 1939, v127, 437.
9. Matthes, K., Malikiosis, X., *Z. Exp. Med.,* 1943, v112, 476.
10. Cerletti, A., *Helv. Physiol. Acta,* 1945, v3, 537.
11. Steinmann, B., Rickenbach, K., Gianoli, A., *Cardiologia,* 1953, v23, 154.
12. Green, H. D., Lewis, R. N., Nickerson, N. D., PROC. SOC. EXP. BIOL. AND MED., 1943, v53, 228.

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Effect of Dicumarol on Bleeding Time.* (24119)

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Roskam(1) reported that heparin has only a slight effect on bleeding time. This observation has been confirmed many times clinically. It is also evident from simple clinical observation that the newer indirect anticoagulants such as dicumarol have little effect on normal bleeding. However, there has not been any recent investigation of the effect of dicumarol on bleeding time. Roskam showed that the effect of heparin on bleeding time depended on technical details of method used. In our study, we used the original method of Roskam and Pauwen(2). Link(3) reported that a certain number of normal rabbits fail to show increase in prothrombin time with dicumarol (non-reactors). This provides a useful control in many experiments on dicumarol (4). As reported separately(5), administration of ACTH to rabbits results in rabbits previously refractory to dicumarol showing elevated prothrombin times with the drug. One series of our rabbits were therefore treated with ACTH in addition to dicumarol.

Methods. Bleeding times were determined on ear of rabbit by the method of Roskam and Pauwen(2). The ear was shaved, the rabbit immobilized. Ten cuts were made on each ear and the ears bathed with distilled water at 30°C and pH 7.0. Time for bleeding to stop from each cut was recorded and mean bleeding time with standard deviation calculated. Prothrombin times were done by the usual Quick procedure using rabbit brain thromboplastin. Dicumarol was given orally as a single dose of 5 mg/kg. ACTH was given to some rabbits as a single intramuscular dose of 5 mg/kg at the same time as dicumarol.

Results. Eighteen rabbits were given dicumarol and prothrombin times and bleeding times determined before (day 0) and on the third day. Five rabbits were similarly treated and prothrombin times and bleeding times determined before and on the fifth day. Eleven rabbits received both dicumarol and ACTH and prothrombin times and bleeding times determined before and on fifth day. The results are shown in Table I. P values are for value of mean bleeding time compared to the same value on day 0. Comparing bleeding time on third day with that at beginning of experiment, the average of mean bleeding times for the 18 rabbits showed a slight but significant increase. Eight of the 18 rabbits gave a sig-

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TABLE I. Effect of Dicumarol on Prothrombin Time and Bleeding Time.

Dicumarol alone						Dicumarol alone					Dicumarol and ACTH					
Prothrombin time (sec.)		Mean bleeding time (sec.)			Day of	Prothrombin time (sec.)		Mean bleeding time (sec.)			Prothrombin time (sec.)		Mean bleeding time (sec.)			Day of
Day 0	Day 3	Day 0	Day 3	P	death	Day 0	Day 5	Day 0	Day 5	P	Day 0	Day 3	Day 0	Day 3	P	death
11.2	>480	136 ± 7.2	107 ± 13.3	<.1	4	8.3	11.0	170 ± 11.2	151 ± 44.7	>.5	23.6	66.5	135 ± 7.9	*199* ± 31.6	<.01	7
12.4	"	129 ± 4.4	*193* ± 15.1	<.01		8.6	8.3	193 ± 21.2	182 ± 25.9	"	9.5	44.2	184 ± 10.8	*274* ± 35.9	<.05	6
10.2	219	136 ± 13.6	146 ± 21.6	<.5		10.0	10.2	170 ± 17.0	155 ± 25.3	"	25.8	276	146 ± 7.0	177 ± 19.0	<.2	
9.8	>480	126 ± 12.9	154 ± 26.5	<.4	4	8.6	9.5	178 ± 17.1	161 ± 23.0	"	14.8	>480	212 ± 28.4	145 ± 16.5	<.1	
9.3	"	135 ± 8.4	129 ± 7.2	<.5	"	11.0	9.7	170 ± 20.1	183 ± 39.2	"	10.1	61.8	180 ± 31.2	192 ± 31.5	>.5	
11.2	234	128 ± 8.8	*163* ± 12.5	<.05	"						10.8	50.2	198 ± 22.0	181 ± 16.8	"	
8.6	13.3	134 ± 9.6	*223* ± 29.5	<.02				M: 176 ±	166 ±	"	9.7	43.1	193 ± 10.8	161 ± 15.7	"	
9.3	309	126 ± 21.8	196 ± 27.3	<.1							9.6	314	151 ± 15.9	188 ± 5.8	<.1	
10.1	51.2	155 ± 30.7	119 ± 8.4	<.3	4						10.1	33.1	147 ± 12.7	174 ± 14.0	<.2	6
15.2	348	142 ± 11.1	156 ± 21.7	<.5							27.3	238	164 ± 20.9	*296* ± 22.9	<.01	
9.5	323	120 ± 3.9	*204* ± 9.7	<.01	12						8.9	>480	206 ± 19.7	152 ± 6.3	<.05	
9.9	17.2	148 ± 19.2	*220* ± 12.6	<.01												
10.6	22.8	126 ± 17.7	168 ± 25.0	<.2								M:	170 ± 8.4	194 ± 19.4	<.2	
8.4	97.2	124 ± 10.4	*262* ± 45.2	<.02	4											
10.2	>480	126 ± 14.9	107 ± 8.9	.3	"											
26.4	"	156 ± 8.8	*225* ± 21.2	.01	6											
10.6	15.8	156 ± 4.7	*218* ± 23.2	<.05												
9.6	17.4	135 ± 12.6	173 ± 22.8	.3												
	M:	135 ± 4.2	176 ± 10.6	<.05												

Mean values showing significant difference from those for day 0 are italicized.

nificantly greater bleeding time on third day. Three of the 4 non-reactor rabbits which showed only a slight increase in prothrombin time increased in bleeding time. On the other hand, 9 rabbits showed no increase in bleeding time, and 7 of these had quite prolonged prothrombin times. Six rabbits showed an increase in both values. One animal with increased prothrombin time showed a significant decrease in bleeding time. There was therefore little relationship between the 2 values.

There have been reports of dicumarol affecting vessels directly(6). The mean bleeding time and prothrombin time were determined for 5 non-reactor rabbits on fifth day after receiving dicumarol. There was no change in bleeding time. As found previously, when dicumarol and ACTH were administered simultaneously to rabbits, all animals showed increase in prothrombin time. Three of these rabbits gave an increased bleeding time 3 days after receiving dicumarol and ACTH. This incidence was no greater than with dicumarol alone and the other rabbits were unaffected. One animal showed a significant decrease in bleeding time. On the other hand one rabbit died of hemorrhage without increase in bleeding time from ACTH and dicumarol.

A most remarkable finding was that the animals on which bleeding time determinations were performed on third day and in all of which the bleeding had stopped by 5 minutes after the cut, showed bleeding commencing again 1-2 hr later. Eight of these animals were dead the next morning in their cages and 4 died in the next 3 days. Some small amounts of blood were present in the cage and on the fur. When the animals were autopsied all organs were found to be exceedingly pale and anemic. Lung pathology was observed in 4 rabbits. The general picture was of extensive hemorrhage and shock although large amounts of blood were not found in the cage. The picture was identical with that already observed by Jaques *et al.*(7,8) for rabbits on anticoagulants subjected to stress. It appears to be due to a non-specific hemorrhage or leaking of blood into tissues all over the body without any particular area of specific hemorrhage. It is particularly remarkable that rabbits which had a perfectly normal bleeding time at 2 p. m. were dead from hemorrhage the next morning.

Discussion. That animals bleed to death internally following determination of bleeding time externally, even though bleeding time is normal, is a remarkable observation. It can be explained on the basis of previous observations of Jaques *et al.* that when rabbits on dicumarol are subjected to stress, death from hemorrhage occurs in a high percentage of animals. Determination of bleeding time by repeated cutting of the rabbit ear seems to be an effective stress stimulus in the rabbit as judged by this high mortality. There is no increase in mortality when ACTH is added. As reported separately(5), administration of ACTH instead of stress to dicumarolized rabbits also results in hemorrhagic death in rabbits. Bounameaux, van Cauwenberge and Roskam(9) demonstrated that the immediate effect of ACTH and cortisone was to shorten bleeding time of rabbits. This is also illustrated by the fact that there was no increase in bleeding time with dicumarol plus ACTH and indicates that bleeding time is not a measure of the changes in vessels due to ACTH.

Summary. Rabbits were treated with dicumarol and dicumarol plus ACTH and prothrombin times and bleeding times determined. A slight increase in bleeding time was observed after 3 days on dicumarol, in 50% of the rabbits. Half the rabbits died in the next 24-72 hr from generalized non-specific hemorrhage. This was not related to the previous increase in bleeding time. Bleeding did recur and continue from the cut ear but hemorrhage was much more extensive than this. It is suggested that determination of bleeding time is a marked stress procedure in rabbits.

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1. Roskam, J., *Arch. Int. Pharmacodyn.*, 1942, v68, 66.

2. Roskam, J., Pauwen, L., *ibid.*, 1937, v57, 450.

3. Link, K. P., *Harvey Lecture,* 1943-4, v39, 162.

4. Jaques, L. B., Froese, E. Lepp, O'Toole, R. E., Spinks, J. W. T., *Arch. intl. de pharmacol.*, 1957, III, v4, 478.

5. van Cauwenberge, H., Jaques, L. B., *Can. Med. Assn. J.*, in press.
6. Dale, D. U., Jaques, L. B., *ibid.*, 1942, v46, 546.
7. Lepp, E., Chubaty, W., Jaques, L. B., *Can. J. Med. Sc.*, 1953, v31, 173.
8. Jaques, L. B., Mogenson, G. J., Fisher, L. M., *Trans. Roy. Soc. Can. Third series*, 1956, v50, Sec. V, 9.
9. Bounameaux, Y., van Cauwenberge, H., Roskam, J., *Schweiz. Med. Wchschr.*, 1954, v84, 789.

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Purification of Erythropoietin by Ion-Exchange Chromatography.* (24120)

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We reported(1,2,3) that erythropoietic activity of acidified, boiled plasma filtrates prepared from phenylhydrazine anemic rabbits was associated with a protein having paper electrophoretic mobility of an alpha globulin and chemical characteristics of an acid glycoprotein. Small molecular weight, acidic glycoproteins are found in Cohn Fraction VI(4). In preliminary work with plasma from phenylhydrazine anemic rabbits and from a patient with polycythemia vera we found erythropoietic activity in Fraction VI (unpublished). The present study was therefore designed to isolate, by ion-exchange chromatography, acidic glycoproteins from the crude source of boiled anemic rabbit plasma.

Materials and methods. Acidified, boiled plasma filtrate (APF) obtained from phenylhydrazine anemic, adult, white male rabbits was prepared by modification of method of Gordon(5) as previously described(3). The filtrate was dialyzed in the cold 48 hours against repeated changes of distilled water and then lyophilized. One liter of plasma yielded 500 to 600 mg of lyophilizate. The lyophilizate, which possesses erythropoietic activity by our previously described method (3), was fractionated on diethylaminoethyl (DEAE) cellulose ion-exchange columns(6). Two separation procedures (I,II) were utilized. Procedure II was designed for a more definitive fractionation on the basis of results obtained with procedure I. The separation experiments are summarized in the elution diagrams in Fig. 1 and 2. Fraction pools as designated in Fig. 1 were dialyzed, lyophilized and redissolved in 0.9% NaCl. Along with unmodified APF starting material, and control saline injections, these fractions were assayed for erythropoietic activity. Fraction pools as designated in Fig. 2 were assayed directly, using a solution of 0.9% NaCl in 0.01 M sodium acetate buffer as a control injection. Female, Sprague-Dawley rats (average weight, 220 g) were injected subcutaneously daily for 4 days with 2 mg by dry weight (in 2 ml 0.9% NaCl) of the respective fractions from procedure I. The fractions from procedure II were injected on a similar schedule, with a daily dose of 0.5 mg by protein content. Protein content was estimated on the effluent fraction pools by the ratio of optical density measurements at 280 and 260 mμ according to the method of Warburg and Christian(7). On day 4, the animals were given an intravenous injection of 0.5 μc iron-59 as iron citrate contained in 0.25 ml of solution. The per cent of injected dose of iron-59 appearing in the total red cell mass in 24 hours was estimated by standard scintillation counting technics on whole blood obtained by aortic exsanguination under ether anesthesia. The total red cell mass was calculated from the microhematocrit assuming an average blood volume of 5.18 ml/100 g body weight(3). Prior to sacrifice, blood from tail vein was obtained for reticulocyte enumeration by the dry cresyl-blue method.

Results. Erythropoietic activity of isolated

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