man subjects, the diabetes was well controlled at the time of observation.

Reductions in serum mucoprotein have been most frequently associated with impaired liver or endocrine function(13,14); our finding is a decrease in the protein bound hexosamine. Since the liver has been implicated as the primary site of glucosamine formation(15), it may be possible that these alloxanized rats have impaired liver function, resulting in a decreased synthesis of these compounds. The decreases in serum proteins, particularly α_1 globulin and albumin fractions in diabetic rats observed by Nichols *et al.*(10) are indicative of altered hepatic activity.

It has been reported that the liver of the diabetic animal as the primary site of glucosamine formation can synthesize hexosamines at a rate similar to the normal animal (5). If this were the case, the lower levels in the diabetic rat may be a reflection either of decreased synthesis or release of these compounds by secondary sites, such as connective tissue.

Summary. In both cells and plasma, alloxanized rats had significantly lower concentrations of protein-bound hexosamine than normal animals. In diabetic patients under insulin control, values were normal. Concentration of protein-bound hexosamine was twice as high in plasma and erythrocytes of normal rats as in normal human beings.

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1. Winzler, R. J., *Methods of Biochemical Analysis,* Vol. 2, Interscience Publishers, Inc., New York, N. Y., 1955, p279.

2. Jacobs, H. R., *J. Lab. Clin. Med.,* 1949, v34, 116.

3. Berkman, J., Rifkin, H., Ross, G., *J. Clin. Invest.,* 1953, v32, 415.

4. Shetlar, M. R., Foster, J. V., Kelly, K. H., Shetlar, C. L., Bryan, R. S., Everett, M. R., *Cancer Res.,* 1949, v9, 515.

5. Spiro, R. G., *Ann. N. Y. Acad. Sci.,* 1959, v82, 366.

6. Schiller, S., Dorfman, A., *J. Biol. Chem.,* 1957, v227, 625.

7. Elson, L. A., Morgan, W. T. J., *Biochem. J.,* 1933, v27, 1824.

8. Boas, N. F., *J. Biol. Chem.,* 1953, v204, 553.

9. Rosenlund, B., *Scand. J. Clin. and Lab. Invest.,* 1956, v8, 343.

10. Nichols N., Moinat, P., Tuller, E. F., *Diabetes,* 1956, v5, 468.

11. Mills, F. C., *Statistical Methods,* New York, N. Y., 1938.

12. Greenspan, E. M., *Arch. Int. Med.,* 1954, v93, 863.

13. Greenspan, E. M., Tepper, B., Terry, L. L., Schoenbach, E. B., *J. Lab. Clin. Med.,* 1952, v39, 44.

14. Greenspan, E. M., Lehman, I., Graff, M. M., Schoenbach, E., *Cancer,* 1951, v4, 972.

15. Spiro, R. G., *J. Biol. Chem.,* 1959, v234, 742.

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Different Hemostatic Responses of Dogs to Heparin Injection.* (26594)

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It is well known that the only effect which heparin has on hemostasis when it is injected intravenously in humans as in thrombosis is a drastic one on coagulation. The occurrence of rare accidents in heparinized patients has been interpreted as an individual susceptibility to hemorrhage while the blood remains incoagulable.

In our extensive observations on skin hemostasis in dogs, before and after injection of variable amounts of heparin, marked differences were noted not only with varying amounts of heparin, but also with a standard dose in different dogs. This difference in group response in dogs is described herein.

Methods. Unless otherwise stated the he-

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TABLE I. Group Incidence of Skin Hemostasis Variation in Dogs after Intravenous Injection of Heparin (300 I.U./kg).

Heparin dog group	Bleeding time (min.)	Sex ♂ No.	♂ %	Sex ♀ No.	♀ %	No. not observed	Total No.	Total %
Resistant	5-12	254	63	194	59	57	505	63
Intermediate	13-29	75	18	67	20	4	146	18
Labile	30 or more	79	19	68	21	2	149	19
Total		408	—	329	—	63	800	—

parin employed in this investigation was Liquemin, Roche (5,000 I.U. per ml), diluted with saline and injected slowly intravenously. Other brands were Evans, Connaught, and Lutetia. Both anesthetized (sodium pentobarbital) and unanesthetized dogs were studied. Skin hemostasis was observed by the determination of bleeding time from safety razor blade cuts made, usually in the inner portion of the thigh, 10 minutes after the heparin injection, according to the technic previously described(8,9). Coagulation time was determined by the method of Lee and White(18). Heparin units refer to international units in which 100 I.U. are approximately equivalent to 1 mg of the barium salt of heparin.

The bleeding time of normal, untreated dogs varies from 5 to 11 minutes, with values of greater than 12 minutes very rare. Thus in this study the dogs, after a standard dose of 300 I.U. of heparin per kg, were divided into 3 groups: I, heparin-resistant dogs with bleeding times of less than 13 mins; II, heparin-intermediate dogs with bleeding times of greater than 13 minutes, but less than 30 minutes; and III, heparin-labile dogs with beeding times of more than 30 minutes.

Results. Incidence of a heparin effect. Table I shows the incidence of skin hemostasis disruption after intravenous administration of 300 I.U. of heparin per kg. The incidence is the same in both sexes, and if we consider all abnormal bleeding times, heparin administration at this dosage level interfered with the mechanism for skin hemostasis in slightly more than one-third of all dogs (37%).

Effect of anesthesia and surgical procedure. Bleeding and coagulation times in a heparin-labile dog which had received 580 I.U. of heparin per kg increased to more than 30 minutes. Anesthesia induced by sodium pentobarbital one hour after the heparin injection and continued for 3 hours did not alter either bleeding or coagulation time. After natural elimination of heparin (1.5 hr) and when both times had returned to normal levels, thoracotomy was performed and heparin once more injected (425 I.U. per kg). Bleeding and coagulation times rose to more than 30 minutes. One and one-half hours later both times again returned to normal. In 8 heparin-intermediate dogs the post-heparin bleeding times were 16, 20, 14, 16, 16, 21, 19, and 18 minutes before anesthesia. After anesthesia these values were 14, 40, 20, 16, 19, 28, 18, and 16 minutes, respectively.

Effect of different brands of heparin. Different brands of heparin were injected successively into a heparin-labile dog after bleeding and coagulation times had returned to normal following the preceding injection. Liquemin, Roche (B-508011), 425 I.U./kg; Liquemin, Roche (R-505027), 580 I.U./kg; heparin Evans (A-90590), 425 I.U./kg; and Lutetia, 300 I.U./kg in this order, resulted in similar bleeding and coagulation times of greater than 30 minutes.

A heparin-resistant dog received heparin Connaught (Lot 11081), 660 I.U./kg, and Liquemin, Roche (R-505027), 660 I.U./kg. With both Connaught and Liquemin heparin the dog had a normal bleeding time (4-8 minutes), but the blood remained incoagulable for more than 24 hours.

Fat feeding and heparin-lability. In one heparin-resistant and one heparin-labile dog, both of whom received 30 ml of olive oil by stomach tube and heparin intravenously at the peak of lipemia (4 hours after intubation), the coagulation time was greater than

TABLE II. Effect of Intravenous Heparin on Bleeding Time Increase in Heparin-Resistant Dogs.

Dog	Heparin (I.U./kg)	Bleeding time (min.)	Dog	Heparin (I.U./kg)	Bleeding time (min.)
1	300	6	6	300	12
	600	6		600	12
	1200	+30		1200	+30
2	300	10	7	300	9
	900	12		600	9
	1500	16		1200	15
	2100	25		1500	+30
3	300	23	8	300	4
	600	45		600	10
				900	11
4	300	9		1200	+30
	500	9			
	1000	7	9	300	5
	1300	8		600	8
	1700	10		900	13
	2000	23		1200	+30
	2300	+30	10	300	6
				600	+30
5	300	8			
	600	+30	11	300	8
				600	+30

12 hours. However, in the heparin-resistant dog the bleeding time was only 8-9 minutes, while in the heparin-labile dog it was greater than 30 minutes.

Heparin-lability and heparin dosage. Bleeding time increased with increasing dosage in heparin-resistant dogs (Table II). In Table III it is evident that the minimum dosage required to reveal heparin-lability varies considerably from one dog to another.

Persistence of heparin-response in same dog. In 2 dogs heparin-resistance persisted for at least 34 days following the standard dose of 300 I.U./kg. Similarly, heparin-lability persisted for at least 15 days.

TABLE III. Heparin-Lability in Different Dogs as Observed with Variable Amounts of Intravenous Heparin.

Dog	Heparin (I.U./kg)	Bleeding time (min.)	Coagulation time
A	300	+30	+12 hr
	100	11	+12 "
B	300	+30	+12 "
	100	9	+12 "
C	100	+30	22 min.
	50	18	20 "
D	110	+30	+30 "
	60	25	+30 "
	40	20	8 "
	20	10	5 "
E	300	+30	—
	50	19	—
	30	11	—
	0	7	—

Correlation between coagulation and bleeding times. As a crude test of rate of heparin elimination (detoxication), both coagulation and bleeding times were measured in some cases simultaneously. Table IV shows that degree of blood coagulability is independent of either heparin-resistance or -lability.

TABLE IV. Lack of Correlation between Coagulation and Bleeding Time in Resistant and Labile Dogs.

	Dog	Time after heparin inj.* (min.)	Coagulation time (hr)	Bleeding time (min.)
Resistant	1	25	12	8
	2	25	12	10
	3	25	12	8
	4	25	1	7
	5	30	12	9
	6	25	12	6
	7	25	12	6
	8	30	+½	6
	9	30	+½	7
Intermediate	10	45	12	23
	11	40	12	20
Labile	12	50	12	+30
	13	50	+½	+30
	14	45	+½	+30
	15	40	+½	+30

* Heparin in standard dose: 300 I.U./kg.

Discussion. The literature contains reports on the response of bleeding time in man and animals after heparin administration. According to one investigator the bleeding time remains normal in man after therapeutic doses of heparin(19), but another report describes a very small increase of bleeding time in man and rabbits following heparin administration(23). Of 10 rats injected with 500 I.U./kg, hemostasis did not occur in 3 animals. Even with a larger dose of heparin (2250 I.U./kg) hemostasis did not occur in 2 of 4 rats(24). No increase in bleeding time was observed in 5 rabbits who received 880 I.U./kg, in spite of an increase in coagulation time to 30 or 120 minutes(5).

The results described here show that in dogs heparin has, besides its effect on coagulation, the ability to interfere with control of skin hemorrhages, as assayed by bleeding

time determinations. Different commercial brands of heparin are equally active in this regard. We did not observe this in previous work when we reported that either heparin or citrate could be used equally well as an anticoagulant(8). Our previous failure to recognize this phenomenon may have been due to the fact that in this current study we found 63% of 800 dogs to be heparin-resistant, so that only the relatively few earlier observations could well have fallen in this group.

That heparin can induce platelet agglutination *in vitro*(6,7) or *in vivo* with thrombus formation(1-3,22) has been reported. Those workers state that thrombus formation depends upon the strength of the causative agent. Glass tubes, considered as strong agents, used in shunts induce thrombi, and thus keep bleeding time within normal limits. On the other hand, razor blade or needle injuries of the skin would not be strong enough to induce thrombus formation. Thus, according to those authors, prolonged bleeding occurs. This interpretation based on a thrombus as a causative agent in bleeding control when blood was heparinized could not, however, be applied to the phenomenon described herein, since dogs subjected to the same stimulus showed different hemostatic reactions (normal as well as prolonged bleeding times). Other work emphasized, moreover, that agglutination of platelets *in vitro* is related not to the presence of heparin itself, but to contaminating substances from heparin sources in early commercial preparations, since recent heparin products cannot influence platelet agglutination(15). Furthermore, in connection with thrombus formation and bleeding control, experimental studies employing the hind leg preparation for hemostasis studies(10) showed that arterial blood, devoid of platelets by reason of repeated and accurate fractional centrifugation, still keeps its hemostatic properties, casting some doubt on the necessity for the actual presence of a thrombus to induce hemostasis.

The doubt about platelet plugs functioning mechanically in bleeding control is reinforced by studies on the activity of plasma fractions which can convert non-hemostatic venous blood into hemostatic blood(11) or can inactivate arterial blood(12), as well as by observations in other hemostatic fields(13), all of which point to a direct and paramount role of plasma constituents in hemostasis, independent of agglutinated platelets. All of these studies are suggestive of an active plasma substance, acting biochemically rather than by direct mechanical occlusion, in control of hemorrhages of skin, small vessels and capillaries.

There is considerable literature on the effect of heparin on plasma proteins. The electrophoretic pattern of plasma proteins changes with amount of added heparin(17). The effect is very marked at high (0.4%) heparin concentration(4) when a component (Component C) appears to migrate between pure heparin and albumin. Anticomplementary activity of heparin has been studied(14). Plasma constituents involved in the heparin effect are thought to be, in man at least, mainly *beta*-globulin and possibly also *alpha*$_2$-globulin, whereas interaction with *gamma*-globulin and albumin is very slight by comparison(17). The plasma lipoprotein interaction is particularly interesting. It is well known that the clarifying effect of heparin discovered by Hahn(16) is not induced through a direct action of heparin upon plasma lipids but is mediated by a globulin component *in vivo*. Also alteration in the flotation rates of the lipoproteins of serum following heparin administration is detectable by ultracentrifugation(20). The active heparin-containing compound present in tissue mast cell cytoplasm has been extracted and purified(21) showing that heparin is found in a loose linkage with a polypeptide and a lipid which contains cholesterol, lecithin and neutral fats. This lipoprotein part of the molecule is very similar or identical to the heparin co-factor of blood serum according to electrophoretic data and clotting assays.

Our results show that if heparin activity is mediated by formation of a complex "heparin-hemostatic plasma component," this plasma component (rendered inactive by the complex formation) should be present at different concentrations in different dogs be-

cause, to suppress skin hemostasis completely (*i.e.*, a bleeding time of more than 30 minutes), each dog requires a different amount of heparin. Moreover, at least 2 other aspects of this hypothetical component are that there is constant concentration of it in each individual dog, at least for a period of weeks; and that its activity after injection of heparin is not associated with sex of the animal, with anesthesia, with extensive surgical procedures, or with lipemia caused by ingestion of large amounts of olive oil.

With a standard dose of heparin (300 I.U./kg) the distribution of heparin-lability in the dogs is fairly constant, several groups giving results very near the average incidence of 37% with bleeding time greater than 13 minutes. This seems to indicate that distribution of the plasma component rendered inactive by heparin is fixed in the species as a whole, but varies from one dog to another.

As the lability in dogs is quite variable, it is possible that responsiveness to heparin may vary also from one patient to another. Thus, if our findings could be extended to other mammals and to man, they might explain the abnormal bleeding of unknown mechanism that occurs in a percentage of surgical and thrombotic patients under heparin treatment.

Summary. Observations on bleeding time of 800 dogs after administration of a standard dose of heparin (300 I.U./kg) showed different responses which were divided into 3 groups: 63% as heparin-resistant dogs with normal bleeding time up to 13 minutes; 17% as heparin-intermediate dogs with the bleeding time between 13 and 30 minutes; and 18% as heparin-labile dogs with bleeding times higher than 30 minutes. This response of skin hemostasis does not depend on sex, anesthesia, extensive surgical procedures, heparin brand, or lipemia after olive oil feeding. A heparin-resistant or heparin-labile dog remains such after weeks of observation. The extent of lability varies in individual dogs, some showing an increased bleeding time even with comparatively small doses of heparin. On the other hand, heparin-resistant dogs could be made labile with increased amounts of heparin. It appears that heparin interferes with a plasma component normally active in the hemostatic mechanism. These results emphasize the paramount importance of plasma proteins, rather than a platelet thrombus, as the primary factor in control of skin hemorrhage in the small vessels. The possibility of similar findings in humans is stressed and the possible relationship of heparin-lability and some hemorrhagic accidents in heparinized patients is pointed out.

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1. Best, C. H., *Harvey Lectures,* 1940, 41.
2. Best, C. H., Cowan, Maclean, *J. Physiol.,* 1938, v92, 20.
3. Best, C. H., Solandt, D. Y., *Lancet,* 1940, 1042.
4. Chargaff, E., Ziff, M., Moore, D. H., *J. Biol. Chem.,* 1941, v139, 383.
5. Chen, I. I., Tsai, C., *J. Physiol.,* 1948, v107, 280.
6. Copley, A. L., Robb, T. P., *Am. J. Physiol.,* 1941, v133, 248.
7. Copley, A. L., *Fed. Proc.,* 1948, v7, 22.
8. Cruz, W. O., Oliveira, A. C., *J. Appl. Physiol.,* 1958, v13, 368.
9. Cruz, W. O., *The Coagulation of Blood, Methods of Study.* Edited by L. M. Tocantins, Grune & Stratton, 2nd Ed. to be published.
10. Cruz, W. O., Oliveira, A. C., *Brit. J. Haem.,* 1959, v5, 368.
11. Cruz, W. O., Magalhães, J. R., Oliveira, Mecia M., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 740.
12. Cruz, W. O., Magalhães, J. R., Meis, L., *Blood,* 1960, v16, 1433.
13. Cruz, W. O., Studies on Hemostasis *in vivo,* presented in Symposium on platelets. Ford Memorial Hosp. 1960, Detroit. Edited, 1961, by Little, Brown & Co., Boston, Mass.
14. Ecker, E. E., Pillemer, L., *J. Immunol.,* 1941, v40, 73.
15. Fidler, E., Jaques, L. B., *J. Lab. Clin. Med.,* 1948, v33, 1410.
16. Hahn, P. F., *Science,* 1943, v98, 19.
17. Hoch, H., Chanutin, A., *J. Biol. Chem.,* 1952, v197, 503.
18. Lee, R. I., White, P. D., *Amer. J. Med. Sci.,* 1913, v45, 495.
19. MacFarlane, R. G., *Quart. J. Med.,* 1941, v33, 1.
20. Nikkila, E., *Scand. J. Clin. and Lab. Inv.,* 1953, v5, suppl. 8.
21. Snellman, O., Sylvén, B., Julén, C., *Biochem. et Biophys. Acta,* 1951, v7, 98.

22. Solandt, D. Y., Best, C. H., *Lancet*, 1938, v2, 130.

23. Roskam, J., *Arch. Intern. Pharmacodyn. et Thérap.*, 1942, v68, 66.

24. Zucker, Marjorie B., *Am. J. Physiol.*, 1947, v148, 275.

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Metabolism of Tyramine by Smooth Muscle Preparations *in vitro*.* (26595)

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The metabolism of amines, particularly sympathomimetic amines, has been of considerable interest in recent years. In smooth muscle 2 pathways by which these amines are known to be inactivated are oxidative deamination and O-methylation(1,2). Evidence will be presented here for the presence of a new metabolic pathway in smooth muscle which converts p-hydroxy-phenylethyl amines to catecholic compounds. These results were obtained from a study of the metabolism of tyramine by rabbit aorta and stomach muscle.

Materials and methods. Fresh rabbit aorta (descending thoracic and abdominal) was rinsed with buffer, trimmed, and cut into longitudinal pieces. Strips of stomach muscle were prepared by making parallel incisions through the muscle layers only and gently teasing the muscle away from the submucosa. All procedures were carried out at 2-4°C. Incubation flasks (25 ml Erlenmeyer), containing 200 μM oxygenated Na phosphate buffer, pH 7.4, and 20 μM tyramine in a total volume of 2.0 ml, were mounted in a rack and oxygenated by bubbling 100% O_2 below the surface of the fluid for 5 minutes. Strips of tissue were added (100-200 mg wet weight), and the flasks securely stoppered and incubated at 38° with shaking. The incubation was terminated by addition of HCl (final concentration 0.2 N) after removing the tissue (usually after 240 min). Acidified samples were used directly for all chemical and chromatographic analyses. Flasks incubated without substrate or without tissue were included as controls in each experiment.

All spectrophotometric determinations were made with the Beckman DU spectrophotometer fitted with a diaphragm and microcells. For ultraviolet absorption spectra, solutions were read in 0.3 N HCl and 0.3 N NaOH at a dilution sufficient to obtain an optical density of 0.200-0.800.

Procedures used for determination of amine(3) and p-hydroxyphenol(4) groups have previously been described. In the latter method, tyramine (Mann), tyrosine (Fisher), N-methyltyramine (Smith, Kline and French), p-hydroxyphenylacetic acid (Aldrich) and p-hydroxyphenylethanol (synthesized according to Ferber)(5) gave approximately the same molar extinction coefficient(ϵ). p-Hydroxyphenylethanolamine (Winthrop-Stearn), p-hydroxymandelic acid‡ and p-hydroxybenzaldehyde (Eastman) gave about 20% of the color obtained with tyramine. Epinephrine, norepinephrine, cobefrine, 3-hydroxytyramine, and neosynephrine (all from Winthrop-Stearn) gave no significant color under these conditions.

For chromatographic analysis, aliquots of acidified samples (10-2000 μl) were put directly on Whatman #1 filter paper, 5-15 μl at a time. The following solvents were used: 1) n-butanol-glacial acetic acid-water, 4:1:1;

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