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Distribution of Injected Heparin in Different Blood Fractions.* (25128)

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Although the effect of heparin in inhibiting coagulation of blood has been known for a number of years, almost nothing is known with respect to the mechanism by which this action is exerted. It is most probable that at least one step involves an interaction with one of the plasma proteins since the presence of a normal blood component is a necessary requirement in prevention of blood clotting by heparin. As an initial step experiments were, therefore, conducted to determine in which blood fraction the heparin resides subsequent to intravenous administration. When this is decided further studies could be made on the specific fraction involved. We employed heparin-S^{35} since this can be measured in minute amounts. The present report describes experiments on blood fractionation after intravenous injection of heparin and determination of radioactivity in the different fractions.

Methods. Heparin-S^{35} in the form of sodium salt was prepared as described previously(1). This material, having an anticoagulant activity of 152 U.S.P. units/mg, maintained a constant specific activity (8,010 c.p.m./mg) after repeated recrystallization. Other analytical data were as follows: glucosamine 23.22%; nitrogen 2.25%; sulfur 12.02%; sodium 7.88%; $[\alpha]_D^{25}$ + 51°. Three dogs weighing 8-9 kg were injected intravenously with solution of 30 mg of heparin-S^{35} (2.4×10^5 c.p.m.) in physiological saline. Blood samples drawn from animals 15 and 30 minutes after injection were employed in fractionations and subsequent radioactivity assay. First, blood was separated into plasma and cells. A second portion of blood was clotted and serum separated from clotted material was fractionated into components of different densities. After mixing a serum aliquot with aqueous sodium chloride to give a solution with density of 1.006, the mixture was centrifuged at 79,000 g for 20 hours at 4° (Spinco Ultracentrifuge, Model L). The top layer (density, less than 1.006) was removed for radioactivity analysis and the infranate was mixed with salt solution (D, 1.346 to give a final density of 1.063). The latter mixture was centrifuged at 79,000 g for 20 hours and the top layer (D, 1.006-1.063) taken off. The above procedure was repeated with the infranate using potassium bromide as the salt to give top layers with densities of 1.063 - 1.120 and 1.120 - 1.200. The different fractions were then combusted, converted to barium sulfate, and counted in gas flow counter. Another 10 ml of serum was mixed with 90 ml of 5% trichloroacetic acid. The precipitated protein was collected and washed with trichloroacetic acid. The washings were combined with the original supernate. In another experiment serum was mixed with 3 volumes of alcohol and the precipitated material separated from the supernate. Additional experiments were conducted in which plasma was submitted to chemical fractionation by Cohn

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TABLE I. Radioactivity of Blood Fractions after Intravenous Injection of Heparin-S^{35} in Blood Drawn 15 and 30 Min. after Injection.*

Fraction	Radioactivity, cpm/10 ml of blood 15 min.	30 min.
Whole blood	1688 ± 96	1034 ± 82
Cells	76 ± 9	22 ± 3
Plasma	1542 ±116	928 ± 15
Clot	42 ± 2	31 ± 2
Serum	1476 ± 96	841 ± 53
Dialyzed serum	1458 ± 89	830 ± 47
Alcohol precipitate	1452 ±104	810 ± 64
" filtrate	2 ± .6	3 ± 1
T C A precipitate	1402 ± 7	791 ± 19
" filtrate	21 ± 3	8 ± .9

* Based on avg of 3 experiments wherein each experiment was conducted in triplicate.

method IV(2) and the material separated into I + III, II, IV, VI, and albumin fractions. Finally, a serum aliquot was dialyzed against running water for 4 days at 4°, and the dialyzate was assayed for radioactivity. Radioactivity measurements were made on a gas flow counter and sufficient counts were taken to obtain an accuracy of ± 10%. Values obtained were converted to number of c.p.m. at infinite thinness.

Results. Figures obtained on radioactivity assay of the various fractions are a reflection of heparin concentration. This is demonstrated by the fact that radioactivity in blood is dialyzable (Table I). If desulfation and consequent removal of the label had occurred, the latter would be in the form of inorganic sulfate which can pass through the cellophane membrane.

Table I shows results obtained in terms of c.p.m. in various blood fractions. Almost no radioactivity (heparin) is taken up by blood cells and practically all material in plasma can be recovered in the serum fraction. Furthermore, both trichloroacetic acid and alcohol precipitation show that heparin is associated with proteins. Heparin itself, when dissolved in water cannot be precipitated with trichloroacetic acid.

Of special interest is the fact that no significant amount of radioactivity can be recovered in low-density lipoproteins upon centrifugation in media of different density (Table II). This excludes the possibility of association of heparin with chylomicrons and the major portions of α-lipoproteins. The reported effect of heparin on chylomicrons(3) and lipoproteins(4) apparently does not involve any chemical combinations with these substances and must operate by some indirect action. Most probably this involves interaction of heparin with lipoprotein lipase(5) which in turn elicits the lipolytic effect.

Chemical fractionation of plasma and radioactivity assay of different fractions indicates that the major portion of heparin is found in fraction I + III and II (Table III). It is noteworthy that these fractions contain some of the clotting proteins, *i.e.*, fibrinogen and prothrombin, in addition to γ-globulin and β_1-lipoproteins. Whether heparin combines with one or several of the proteins of these fractions cannot be decided from these experiments and our present studies are concerned with this question. The finding that the albumin fraction contained only traces of radioactivity was somewhat surprising since interaction of heparin with albumin has been demonstrated by electrophoretic studies of Chargaff, *et al.*(6). However, it should be noted that experiments in which such binding was demonstrated were conducted with comparatively high concentrations of heparin. In our study and in clinical administration, actual blood heparin concentration is minute. It

TABLE II. Distribution of Radioactivity in Serum Fractions of Different Density. 50 ml of serum from blood drawn 30 min. after injection were employed. (Avg of 3 exp.)

Fraction	cpm in total
Serum	5166 ±320
D, 1.006	13 ± 1
" -1.063	18 ± .6
1.063-1.120	0
1.120-1.200	14 ± 2
1.200	5062 ± 29

TABLE III. Radioactivity in Plasma Fractions after Chemical Fractionation. 10 ml of plasma obtained from blood 30 min. after injection.

Fraction	Radioactivity, cpm
Original plasma (10 ml)	928 ± 15
Fraction I + III	606 ± 39
II	93 ± 10
IV	14 ± 1
VI	16 ± 3
Albumin	8 ± 1

is quite possible, therefore, that interaction of heparin with albumin does not occur under these conditions.

Heparin is a highly charged molecule and it is reasonable to expect that the mechanism for its actions involves some protein interaction through these charges. Although studies have demonstrated interaction of heparin with individual proteins, comparatively little is known of how it reacts when administered in the bloodstream. The present study narrows the possibilities of reaction to the proteins of certain specific fractions.

Summary. 1. After intravenous injection of heparin-S^{35}, radioactivity is concentrated in the serum fraction. Only minute amounts of radioactivity are found in low density lipoproteins. Chemical fractionation shows that the preponderant portion of radioactivity is in Cohn fractions I + III, lesser amounts in fraction II and only minute quantities in other fractions. 2. Heparin is not desulfated in the bloodstream.

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Development of a Canine Globulin Concentrate. (25129)

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Antiserum prepared from blood of dogs hyperimmunized against distemper has been used to aid in controlling this disease since 1925. More recently, antigens of *Leptospira canicola* and infectious canine hepatitis virus have been added to the hyperimmunizing antigens to make available a single antiserum containing specific antibodies against each of these major diseases. In some cases antigens of common bacterial secondary invaders also are used. Multivalent antiserums prepared in this manner have proved valuable for conferring passive immunity for immediate protection against the agents used for hyperimmunization, and as a part of therapeutic programs after exposure. Presently available antiserums have 2 outstanding disadvantages: (1) antibody content is not precisely standardized, and (2) they contain large amounts of immunologically inert proteins. A degree of standardization may be attained by using large numbers of donor dogs and bleeding repeatedly so that turnover in donor population is gradual and influences antibody content only slightly. Advances in immunochemistry have made purification and concentration of antibody proteins possible, as well as more precise methods of assay. Such improvements were instrumental in developing gamma globulin in human medicine. They also have provided means for developing similar preparations in veterinary medicine. This report is concerned with development of a purified canine globulin concentrate, derived from blood of hyperimmunized dogs, and presents results obtained with this concentrate.

Methods and materials. Starting material used for preparation of globulin concentrates was plasma obtained from pooled blood of hyperimmunized adult dogs. The hyperimmunizing process involved weekly injections of canine distemper virus, infectious canine he-