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-

patitis virus, *Leptospira canicola*,* and a mixed bacterin prepared from cultures of *Salmonella typhimurium*, *Brucella bronchiseptica*, and *Streptococcus* sp. Each lot was prepared from plasma of at least 100 donor dogs. Blood was collected into sodium citrate solution, and the plasma separated by centrifugation. Plasma was defibrinated by first heating at 54°C for one hour and cooling rapidly. Fibrin was removed from the serum by passing through coarse screens and centrifuging. To the clarified serum, at pH 6.8, methanol was added to final concentration of 25% while temperature of the mixture was brought to −6°C. A precipitate which formed was separated from the supernate by Sharples centrifugation. This precipitate contained most of the beta and gamma globulins, while albumins and most other serum proteins remained in the supernatant fluid. The precipitate was freed of alcohol by drying from the frozen state. After desiccation, the dried powder was restored to one-fifth the volume of the original serum and filtered to remove bacteria. Animal protection tests were conducted in littermate puppies, except in a few instances when hamsters were used for studies with *Leptospira canicola*. Litters of puppies were purchased from farms when 10 to 20 weeks of age, and immediately removed to isolation quarters. Isolation units were constructed of concrete and cement plaster so that they could be thoroughly disinfected and cleaned with hot water, and were supplied with filtered air under continuous positive pressure. Titrations comparing whole serum pools (parent serums) and their globulin concentrates were carried out in single litters of puppies. Parent serums and purified globulin concentrates were administered in measured volumes/lb of body weight. Replicate tests with various lots were made in several litters of dogs to determine respective minimum protective dosages. Serum or its purified globulin concentrate was given subcutaneously 24 to 48 hours prior to challenge with the disease agent under study. Dogs challenged with canine distemper virus were exposed both by parenteral inoculation of virulent virus, and by contact with distemper-infected dogs. For this challenging procedure dogs were removed from isolation pens and taken to kennels used for production of canine distemper virus. Here they were injected subcutaneously with 1 to 2 cc of a suspension of spleen tissue containing virulent virus, after which they were placed in pens for 6 to 24 hours with dogs previously infected with canine distemper. They were then returned to isolation units, observed for 21 days, and daily temperature records kept. Whenever clinical observations were inconclusive with respect to canine distemper infection, a complete postmortem and histopathologic examination of the dog was made when the observation period terminated. Challenge with infectious canine hepatitis virus was by intravenous injection of tissue culture fluids with high concentrations of virulent infectious canine hepatitis virus. The challenge virus was isolated from a dog in the terminal stage, and carried in tissue culture for only a few passages. Dogs challenged with infectious canine hepatitis virus were observed 14 days, during which daily white blood cell counts and temperature recordings were made. Challenge with *Leptospira canicola* was carried out by intraperitoneal inoculation of strains highly virulent for the test species (dog or hamster). Test animals were observed 14 days after challenge. Susceptible dogs showed typical symptoms of leptospirosis and usually became severely icteric, while susceptible hamsters usually died within 10 days. In testing antiserums and their concentrates for protective potency against exposure to the various diseases, chief emphasis was placed on protection against canine distemper virus. This course was followed for 2 reasons: (1) canine distemper is the most widespread and difficult to control of the major specific infections of dogs, and (2) smaller amounts of serum or concentrate protect against infectious canine hepatitis or leptospirosis than against canine distemper. The various lots of parent serums and concentrates also were tested serologically for antibody content. Neutralization titers against canine distemper virus were determined by procedures generally following those described by Baker, Gorham and Leader(1).

* Cultures stored in frozen state and thawed just before use.

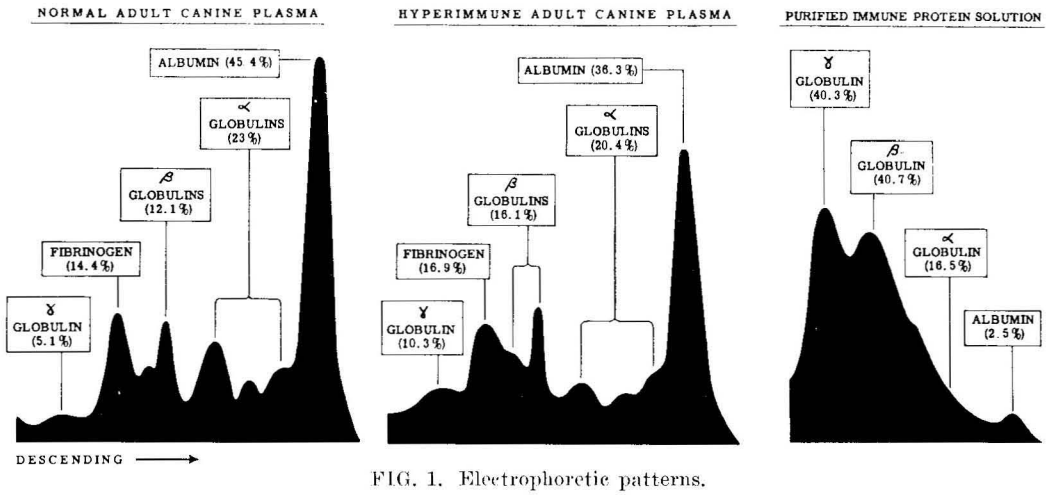

FIG. 1. Electrophoretic patterns.

Dilutions of serum or concentrate were mixed with uniform amounts of an egg-adapted test strain of canine distemper virus, incubated, and the mixtures inoculated on the chorioallantoic membranes of developing chick embryos. Presence or absence of typical lesions after incubation indicated the neutralizing capacities of various dilutions of serum. Infectious canine hepatitis virus neutralization tests were made in trypsinized dog kidney tissue culture systems as described by Cabasso and associates(2) and Fieldsteel and Emery (3). Leptospira antibodies were measured by the agglutination-lysis technic described by Coffin and Stubbs(4). Paired blood samples taken from dogs before and after injection of serum or globulin concentrate also were tested to determine how much passive antibody appeared in the blood of the recipient.

Results. Early in the study, pure gamma globulin of hyperimmune canine serum was found to contain only about 50% of the total antibody, and all of the antibody was recovered only if both gamma and beta globulin fractions were included. Electrophoretic analysis showed that concentrate obtained by the method described is a solution of proteins, 80% or more of which consist of gamma and beta globulins. Fig. 1 shows typical electrophoretic patterns of normal canine serum, hyperimmune canine serum, and globulin concentrate of hyperimmune canine serum.

Comparative studies showed that amounts of beta and gamma globulins present in parent hyperimmune serum pools and their purified concentrates were quite consistent, and antibody titers of either material tended to follow total concentrations of beta and gamma globulins (Table I).

The results of challengng 132 dogs in 20

TABLE I. Antibody Titers and Globulin Contents of Hyperimmune Canine Serum and Globulin Concentrates.

Lot	Product	Globulin content, mg/ml	Canine distemper titer*	Infectious canine hepatitis titer†	*L. canicola* titer‡
1	Globulin conc.	119	46,000	15×10^4	4096
	Hyperimmune canine serum	29	11,000	$5 \times$ "	1024
2	Globulin conc.	104	31,000	$15 \times$ "	4096
	Hyperimmune canine serum	21	10,000	$5 \times$ "	2048
3	Globulin conc.	102.5	36,000	15×10^5	4096
	Hyperimmune canine serum	21.5	6,000	15×10^4	512

* ND_{50}/1 cc determined by egg serum-virus neutralization test at level of 112 EID_{50} of virus /egg dose.

† ND_{50}/1 cc determined by tissue culture serum-virus neutralization test at level of 1000 $TCID_{50}$ of virus/tissue culture dose.

‡ Reciprocal of highest dilution giving a 2+ reading as determined by agglutination lysis test.

TABLE II. Determination of Protective Dosage (50%) and Globulin Content of Hyperimmune Serum and Concentrate.

Dosage	Canine distemper		Infectious canine hepatitis		*Leptospira canicola*	
	Results of challenge	50% protective dose mg globulin	Results of challenge	50% protective dose mg globulin	Results of challenge	50% protective dose mg globulin
Globulin concentrate		4.3		.96		3.8
1.0	7/7					
.5	8/8					
.2	6/7		2/2			
.1	7/7		"		2/2	
.05	3/4		1/1		1/1	
.025	1/4		"		0/2	
.0125			2/2			
.00625			0/1			
Hyperimmune serum		3.8		1		<2.9
1.0	4/4		2/2			
.5	11/11				2/2	
.25	4/4		1/1		1/1	
.125	1/4		"		2/2	
.0625			2/2			
.03125			0/1			
Controls	2/28		0/10		1/4	

Denominator = No. of dogs tested; numerator = No. of dogs remained well.

litters are summarized in Table II. Many other tests are not included because the littermate control puppies either were immune, or failed to react typically to challenge.

While 50% protective dosage volumes of concentrates and parent serums are very different (Table II), each contains approximately the same amount of gamma and beta globulins/dose, indicating complete recovery of antibody with the beta and gamma globulin fraction.

Protective endpoints of the 3 lots of concentrates and parent serums against canine distemper virus compared rather closely in terms of mg of globulin (Table III). While the 50% endpoint results shown in Table III indicate some difference in antibody recovery between Lots 2 and 3, the number of dogs involved in testing individual lots may be too small to consider the difference reliable. However, there were minor variations in the defibrination process, and clarification and filtration of Lot 2, while Lot 3 was prepared exactly as outlined previously.

In addition to tests in dogs for determining relative potency of antiserum and concentrate against *Leptospira canicola,* concentrate Lots 1 and 2 and their parent serums also were

TABLE III. Comparative Protective Doses for Canine Distemper and Their Globulin Content.

Dosage (cc/lb)	Lot #1		Lot #2		Lot #3	
	Results of challenge	50% protective dose mg globulin	Results of challenge	50% protective dose mg globulin	Results of challenge	50% protective dose mg globulin
Globulin concentrate		<3.0		5.2		3.6
.2	2/2		4/5			
.1	1/1		5/5		1/1	
.05	"		1/2		"	
.025	"		0/2		0/1	
Hyperimmune serum		5.2		2.7		3.8
1.0			4/4		1/1	
.5	1/1		9/9		"	
.25	"		2/2		0/1	
.125	0/1		1/2			
Controls	1/7		0/18		1/3	

Denominator = No. of dogs tested; numerator = No. of dogs remained well.

TABLE IV. Protection Tests with Hyperimmune Canine Serum and Globulin Concentrate in Hamsters. Challenging agent was *Leptospira canicola*.

	Lot #1		Lot #2	
Dosage = 0.3 cc of dilution indicated	Results of challenge	50% protective dilution	Results of challenge	50% protective dilution
Globulin concentrate		1:64		1:45
1:4	4/4		4/4	
1:8	"		3/4	
1:16	"		4/4	
1:32	3/4		3/3	
1:64	"		1/4	
Hyperimmune serum		1:12		1:7
1:4	3/4		3/4	
1:8	4/4		2/4	
1:16	1/4		0/4	
Controls	0/3		0/3	

Denominator = No. of hamsters; numerator = No. protected.

tested in hamsters. These tests indicated a somewhat greater difference in hamsters between protective doses of parent serums and their concentrates (Table IV).

Antibody concentrations demonstrable in serums of littermate puppies which had received identical doses of antibody/lb. body weight sometimes varied considerably (Table V). However, at higher dosages, puppies were resistant to challenge with virulent canine distemper virus whether their serum titer after administration of antibody was high or low. At lower dosages, some puppies with no demonstrable serum antibodies resisted challenge with virulent distemper virus, whereas some puppies with low titers developed distemper.

Discussion. That the active fraction can be separated from hyperimmune canine serum in highly purified form without significant loss or destruction of antibody is of considerable clinical importance. Comparatively, purified globulin concentrates, like parent hyperimmune serums, are effective in smaller dosages for protection against *Leptospira canicola* or infectious canine hepatitis than against canine distemper.

Purification of the antibody carrying fraction of serum opens the way for standardization of antibody content by standardizing the amount of globulin contained in unit volumes of purified concentrate. In this way, variations in concentration of antibody in serum pools can be nullified, whereas concentration based on volume alone would cause each lot of concentrate to reflect variations between lots of parent antiserums. Knowing the antibody globulin content of the concentrate, and minimum average dose for short-term passive protection, it would be possible to administer antibody in multiples of the minimum dose for special purposes in more precise dosage than has heretofore been possible.

That resistance to distemper correlated well with amount of antibody administered/lb. body weight, but less precisely with the level of antibody demonstrated in the blood, indicates that passively acquired antibody may be active in tissues and body fluids other than blood.

In addition to the demonstrated immunizing activity of concentrated globulins in these experiments, field trials may well be expected to demonstrate a wider usefulness. In commercial lots, bleedings from 1000 or more dogs, originating over a wide area, are incorporated in each pool of plasma. It is assumed that, in addition to antibodies resulting from specific hyperimmunizations, the purified globulin concentrates may contain other antibodies normally present in adult canine populations exposed to various infectious agents. While they would be present also in parent serums, their concentration before processing would be so low that protective doses would be unreasonably large for practical use. As with human gamma globulin(5), the concentrated canine globulin might make significant amounts of such common antibodies available in practical dosage. If the concentrate could

TABLE V. Canine Distemper Antibody Titers after Various Dosages of Globulin Concentrate or Hyperimmune Serum, and Results of Challenge with Virulent Distemper Virus.

Litter	cc/lb	CD antibody titers* Preinoc.	24 hr postinoc.	Results of challenge
	Globulin conc.			
I	1	Neg.† 1:10	260	Remained well
"	"	" "	220	*Idem*
H	"	" "	310	"
"	"	" "	220	"
I	"	" "	220	"
"	"	" "	150	"
H	"	" "	220	"
"	"	" "	180	"
I	.2	" "	<100‡	"
"	"	" "	"	"
H	"	" "	Neg. 1:10	"
"	"	" "	" "	"
L	.1	" "	" 1:5	"
M	"	" "	" "	"
L	.05	" "	" "	"
M	"	" "	<50‡	"
L	.025	" "	" 1:5	CD
M	"	" "	" "	Remained well
	Hyperimmune serum			
L	.5	Neg. 1:10	Neg. 1:5	*Idem*
M	"	" "	50	"
L	.25	" "	" 1:5	"
M	"	" "	" "	"
L	.125	" "	<50‡	CD
M	"	" "	" 1:5	"

Susceptibility to distemper was demonstrated for all litters by characteristic response to challenge of untreated, serologically negative controls.

* ND_{50}/1 cc: Titers determined against following amounts of virus: litters I and H, approximately 100 EID_{50}, and litters L and M, approximately 130 EID_{50}.

† Neg. = no antibody demonstrated at lowest dilution as indicated.

‡ <100 or <50 = some antibody demonstrated at lowest dilution (1:10 or 1:5).

be used in control of other common infections characterized by such symptoms as cough, diarrhea or skin eruptions, it probably would have to be used in larger dosages than those required for control of diseases against which the donor dogs were specifically hyperimmunized. Again, as with human gamma globulin, such possible benefits of purification and concentration of canine globulins can be revealed only after sufficient clinical evaluation.

Summary. A method is described for purifying and concentrating antibody globulins from serum of dogs hyperimmunized against canine distemper, infectious canine hepatitis and *Leptospira canicola*. Our data show that concentrated canine globulins contain all the protective antibody of serum. The method should make possible standardization of antibody content. Possible additional usage of concentrated canine globulins, analogous to clinical usage of human gamma globulin, has been suggested.

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