STUDIES ON THE AGENT OF CANINE VIRUS HEPATITIS (RUBARTH'S DISEASE)

I. COMPLEMENT-FIXATION AND PRECIPITIN TESTS

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I. INTRODUCTION

Rubarth (1947) showed that saline extracts of the liver from dogs infected with canine hepatitis virus would react in the complement-fixation test. If we accept the antigen-antibody reaction as specific, the test should be of value in studies

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of the relationship between mammalian hepatitis viruses about which, as yet, very little is known. These viruses are extremely difficult to study by animal inoculation in the absence of a reliable serological test, because the natural history of the viruses supports the concept of widespread inapparent infections (Rivers, 1948; Parry, Larin & Platt, 1951). In view of this possible application of the complement-fixation technique, the purification and properties of the antigen have been further studied, and the identity of antigens derived from different sources and their specificity have been analysed. In this paper also experiments are described which showed that canine hepatitis antisera will react with the specific antigen in the precipitin test.

The data presented in this paper are part of a wider series of serological investigations made with certain methods developed in these laboratories in an attempt to study the agent of Rubarth's disease.

II. EXPERIMENTAL TECHNIQUE

A. *Materials*

Strains of canine hepatitis virus. These included (1) KEN: Kennett strain; (2) STOCK: Stockholm strain; (3) BECK: Beckenham strain; and (4) SYD: Sydney strain.

Strain of canine para-distemper virus. (5) KENT: Kentford strain.

Sera. Sera were obtained: (*a*) from dogs convalescent after an outbreak of canine hepatitis (Parry, 1950); (*b*) from dogs experimentally inoculated with the virus (Parry *et al.* 1951); (*c*) from dogs hyperimmunized with the virus; and (*d*) from normal young dogs bred at the Station, which had never been deliberately exposed to the virus, and which had never shown any clinical signs of the disease. The sera used were either fresh, or had been frozen soon after collection and stored at $-30°$ C. until required for use.

Saline. 0·85 % NaCl.

Complement. Commercial haemolytic complement or fresh guinea-pig serum.

Haemolytic system. Commercial sheep cell haemolysin was titrated before use. The haemolytic system was prepared by adding an equal volume of saline, containing 10 m.h.d. of haemolytic serum per volume to a 6 % suspension of either fresh or commercial packed sheep cells, quickly mixing and incubating at 37° C. for half an hour.

Aluminium hydroxide. $Al(OH)_3$, B.D.H. laboratory reagent (dry).

Chicken cells. Packed red blood cells from chickens 6–8 months old.

Methanol. CH_3OH, B.D.H. laboratory reagent.

Butanol. $CH_3(CH_2)_3OH$, B.D.H. laboratory reagent.

Ethanol. Anhydrous.

B. *Preparation of antigens*

(1) *By adsorption to, and elution from, aluminium hydroxide*

Aluminium hydroxide was washed in distilled water and made into a 30 % suspension in distilled water. The suspension was sterilized in the autoclave.

Tissues were cut up finely and ground in a mortar with quartz powder and

suspended 1 in 10 in distilled water. These suspensions were frozen several times at −30° C. and thawed by immersion of the tubes in lukewarm water; they were finally thawed until the ice just melted but the fluids were still cold. The thawed suspensions were centrifuged at 3500–4000 r.p.m. to remove coarse particles and to them were then added equal amounts of the aluminium hydroxide suspension. Body fluids were clarified, if neccessary, by centrifugation and mixed with the aluminium hydroxide suspension as described above.

After swirling to obtain thorough mixing, the mixtures with aluminium hydroxide were kept overnight in the refrigerator at 3° C., when the particles of aluminium hydroxide settled to the bottom of the flasks. The supernatant fluids were decanted and the sediments were resuspended in saline at pH 6·8–7·0, which was added in amounts equal to the volumes of the original suspensions or fluids. The saline suspensions were left at 37° C. for 3 hr., with occasional swirling, and the supernatants were then decanted and tested as antigens.

(2) *By adsorption to, and elution from, chicken and sheep cells*

Tissue suspensions in saline or body fluids were prepared as described above. Chicken or sheep cells were then added to the tissue suspensions or fluids to give a 30% concentration. After leaving overnight at 3° C. the suspensions were centrifuged and the red cells resuspended in amounts of saline (pH 6·8–7·0) equivalent to the original volumes. After incubation for 5 hr. at 37° C. the mixtures were centrifuged and the haemoglobin-coloured supernatants collected. These were used as antigens.

(3) *By extraction with organic solvents*

Solid specimens were finely ground up and then made up to 10% suspensions (w/v) with various solvents. When fluid specimens were employed they were mixed with solvents in equal volumes. After stirring thoroughly the mixtures were kept overnight at room temperature. The resulting solvent extracts were separated from the extracted materials which were set aside for control investigations. The solvent extracts of solid specimens were immediately re-extracted at room temperature with equal volumes of saline. Solvent extracts of fluids were also immediately re-extracted with saline but in the proportion of 2 to 1. From limited trials it appeared that the optimal pH for the re-extraction by saline was 6·8–7·0. At the extreme pH values 6·2 and 7·2 extraction was often difficult, because aqueous and non-aqueous phases formed stable emulsions, but the antigenic value of those extracts which could be made at pH 6·2 or 7·2 was similar to that of extracts made at 6·8–7·0. The colourless, slightly opalescent saline extracts of pH 6·8–7·0 obtained after separation by centrifugation from their respective solvents were used as antigens.

(4) *By chemical precipitation*

Preparation of methanol-precipitated antigens. The procedures used in these investigations were similar to those developed by Cox, van der Scheer, Aiston & Bohnel (1947) in the purification of influenza viruses. A 10% tissue suspension

in distilled water was prepared according to the aluminium hydroxide adsorption technique described above. Methanol, cooled to −30° C., was cautiously added to the suspension with constant stirring to a final concentration of 30%, while it was kept in the ice box. The resultant turbid fluid was held at 0° C. (ice) for 2–3 hr. and then centrifuged at 3500–4000 r.p.m. until all the suspended particles were thrown down. The supernatant was removed and the precipitate rinsed with distilled water to remove the remainder of the methanol. Subsequently the precipitate was taken up at pH 6·8–7·0 in a volume of saline equal to that of the original suspension, and after stirring thoroughly was allowed to stay at 0° C. for 24 hr. Any undissolved material was then removed by centrifugation. Fluid specimens were cooled at 0° C. and treated with methanol in exactly the same way. The final supernatant, the methanol-precipitated antigen, appeared water clear.

Preparation of ethanol-precipitated antigens. Ethanol-precipitated antigens were prepared in the same way as methanol-precipitated antigens.

Preparation of butanol-precipitated antigens. Butanol-precipitated antigens were also prepared in the same way, except that, owing to the solvent's solubility in water, butanol was added to give a final concentration of 6%.

C. *Preparation of hyperimmune sera*

Four dogs: DS12, DE25, DE33 and DE26 were hyperimmunized with KEN strain canine hepatitis virus. The immune status of the dogs before the hyperimmunization was as follows: DS12 was convalescent after a natural infection which occurred during an epidemic of canine hepatitis from which the KEN strain was obtained; the other dogs were convalescent after experimental infection with KEN strain.

Dogs were hyperimmunized with clarified virus liver suspension diluted 1:10 in saline. DS12 and DE25 were given eleven intravenous injections of the diluted suspension increasing from 1 to 11 ml. over a period of 3 weeks. DE33 received eleven subcutaneous injections of the virus administered in the same doses and at the same times as those given to the previous dogs. DE26 was given three intraperitoneal injections of 5 ml. of the virus suspension at weekly intervals.

The dogs were bled from the external saphenous vein and the serum was separated from the clot and stored at −30° C.

D. *Complement-fixation test*

Complement-fixation tests were made in test-tubes measuring $3 \times \frac{1}{2}$ in. All sera were inactivated at 56° C. for 30 min. on the day of their use. In brief, the normal scheme of reaction mixtures was as follows:

	ml.
Test serum (or dilution)	0·1
Antigen (or dilution)	0·1
Complement dilution	0·5
Saline diluent to make total volume	1·5

If any component was omitted under any set of given circumstances, enough saline diluent was always added to make the total volume 1·5 ml. Each test-tube of reaction mixture was given primary incubation either in a water-bath at 37° C.

for 30 min. ('hot' complement fixation) or in a refrigerator at 3° C. for 16–18 hr., followed by 1 hr. at room temperature ('cold' complement fixation). Then 0·5 ml. of haemolytic system was added. This was followed by final incubation in a water-bath at 37° C. for 30 min. Readings were then made twice: the first immediately after the final incubation, and the second after the test had been kept at 3° C. for 16–18 hr. 'No haemolysis' was read as + + + +; 'complete haemolysis' as 0; and intermediate degrees as + + +, + +, +. A 'doubtful reaction' was registered as ±.

Complement was titrated before conducting the main test, at first with the haemolytic system alone and then in amounts varying 0·25 m.h.d. in the presence of positive and control antigens as well as positive and normal sera. All tests were carried out with suitable controls as shown below.

When other materials or methods were used they are described separately.

E. *Ring-precipitin test*

Unpurified antigens (dog tissue suspensions or body fluids) were first clarified by centrifugation. As it was rather difficult to clarify some of the crude antigens which were inclined to give non-specific precipitation, water-clear methanol precipitated antigens were subsequently used, prepared as for the complement-fixation test.

Approximately 0·04 ml. of antigen was run very carefully on to the surface of an equal amount of serum in a small test-tube (3 × 25 mm.). If, however, the specific gravity of the antigen was more than that of the serum, as was the case with some crude antigens, the positions of the components were reversed. The reaction was read after the tube had been kept at a suitable temperature for the desired time (see Experimental). A definite ring-like precipitate at the junction of the two liquids was read as a positive result (+). If no such precipitate appeared, the result of the reaction was registered as negative (0). The test was always carried out with controls, namely sera which were known to be positive in the complement-fixation test, normal sera and antigens prepared from the liver of a normal dog.

F. *Quantitative flocculation test*

Only methanol-precipitated antigens were used in this test, which was carried out as follows. The correct dilutions of serum and test antigen were prepared separately in suitable amounts. Each dilution of serum was placed in a number of test-tubes (3 × ½ in.) corresponding to the number of antigen dilutions to be tested. To 0·5 ml. of each dilution of the serum was added 0·5 ml. of the antigen dilution. Each such reaction mixture was maintained for a prescribed time at a suitable temperature (see Experimental). Positive and negative controls were included in the test.

Results were observed with the naked eye in the daylight. Each tube was examined individually, lifted several inches above eye-level and slanted until the fluid spread in a thin layer. A definite turbidity and precipitate was read as a positive reaction (+). Complete absence of turbidity and precipitate was read as a negative reaction (0). Intermediate results were registered as a doubtful reaction (±).

Experience showed that the quality of the serum used for the precipitin test is of great importance. Sera frozen at −30° C. immediately after collection never failed to produce clear-cut results even after 3 months' storage, while the results with sera stored at 3° C. entirely depended upon their age. Stale sera were inclined to give non-specific precipitates even with saline. In order to avoid non-specific precipitation serum preservatives were not used.

III. EXPERIMENTS AND RESULTS

A. *Serological investigations using the complement-fixation test*

(1) *Eluates from aluminium hydroxide*

Antigens from virus-rich dog liver (KEN strain) were prepared by elution from aluminium hydroxide. Antigens from normal dog liver were prepared in the same way for use as controls. All antigens were tested against various sera. No complement fixation occurred with the antigen-eluates from aluminium hydroxide, although the original virus liver suspension gave positive reactions before as well as after the adsorption.

(2) *Eluates from chicken and sheep red cells*

These experiments were designed to demonstrate whether or not antigen particles (KEN strain) could be adsorbed to the red cells. Unfortunately, only one sheep was available as the source of sheep red cells, so that more extensive experiments with this type of cell could not be conducted. The experiments with red cells showed that chicken red cells adsorbed from virus liver suspensions a substance or substances capable of fixing complement in the presence of antisera. These substances were not detectable in eluates from sheep red cells. However, the negative results with sheep red cells were perhaps dependent on variations of individual red cells in their susceptibility to interaction with viruses, as has been recently demonstrated by Sabin & Buescher (1950).

(3) *Solubility of antigen in organic solvents*

To determine the solubility of antigen in solvents, virus liver (KEN strain) and normal liver were extracted with different organic solvents, and the solvent extracts were in their turn extracted with saline. The final saline extracts from respective organic solvents were tested against positive and negative sera. The data shown in Table 1 indicate that although antigen was found in all solvent extracts prepared from virus liver, the bulk of the antigen remained in the sediment after the removal of the solvent extracts. The sediment on being re-extracted with saline at pH 6·8–7·0 produced a very unstable suspension which could be easily separated by centrifugation at 3500–4000 r.p.m. The supernatant, which was an almost colourless, slightly opalescent liquid, was used as the antigen for control of antigenic activity of the sediment after extraction with organic solvent. This antigen produced + + + or + + + + complement fixation with positive sera. It was always possible to reproduce the limited ability of the solvents to extract antigen from various virus specimens used in further investigations (see Table 1).

(4) *Comparative studies of methanol-, ethanol- and butanol-precipitated antigens*

Antigens prepared from different specimens of KEN and BECK strains of virus in such materials as liver, liver fluid, blood and peritoneal fluid taken from affected dogs were compared with control antigens prepared from similar specimens taken from normal dogs. All experiments were repeated at least twice and the results were sufficiently in accord to warrant publication. All antigens prepared by precipitation with the alcohols from virus material were capable of fixing complement with antisera; negative results were obtained with normal sera as well as with the control antigens. However, ethanol and butanol precipitates yielded eluates which were less antigenically active than those obtained with methanol. Because of these results only methanol-precipitated antigens were used in further investigations.

(5) *Reactivity of methanol-precipitated antigens from different virus-rich specimens*

Several experiments were conducted to demonstrate the presence of antigen in the body tissues and fluids of infected dogs. Experiments employing KEN and BECK strains may serve as an example. In the case of KEN strain, body tissues and fluids were harvested from dogs which died from an acute form of naturally acquired virus hepatitis. In the case of BECK strain fluids were harvested from dogs which had been inoculated experimentally (Parry *et al.* 1951). The blood of dog DE 38 was taken in the pre-inoculation period and examined by the complement-fixation test for the presence of antigen in the red cells and the serum, and of antibody in the serum. All these tests gave negative results. Table 2 shows the results obtained with methanol-precipitated antigens from different specimens.

From the data presented thus far, it appears that the behaviour of methanol-precipitated antigens prepared from various virus specimens is quite similar. The KEN strain antigens obtained from the spleen and lungs appeared to be less active than those from other tissues. Subsequent work may show quantitative variations of antigenic material in different tissues of affected dogs.

(6) *Stability of methanol-precipitated antigens*

It seems that the antigenic substance related to canine hepatitis virus is quite stable to frequent changes of temperature in a wide range between + 20 and − 30° C. There was no appreciable difference in yields of methanol-precipitated antigens derived from samples of virus-rich dog peritoneal fluid (KEN and BECK strains) subjected to freezing and subsequent thawing on at least twenty occasions. Methanol-precipitated antigens also appeared reasonably stable to heat, although heating at 100° C. for 1 hr. caused a definite reduction in antigenic activity. A sample protocol of the titration of heated and unheated pooled antigens (serial numbers 107 and 108) which were derived from virus-rich dog peritoneal fluid (KEN and BECK strains) is presented in Table 3.

Storage of methanol-precipitated antigens in a refrigerator at − 30° C. appeared to be satisfactory. After 2 months' storage at this temperature methanol-precipitated antigens had lost very little of their activity.

Table 1. *Complement fixation with different solvent extracts from virus and normal dog livers*

Source of serum		Before extraction		Solvent extracts from virus and normal livers														
				Chloroform		Ethyl ether		Benzene		Petroleum ether		Toluene		Amyl acetate		Butyl acetate		
Dog no.	Immune status	Virus liver	Normal liver	Virus	Normal	Virus	Normal	Virus	Normal	Virus	Normal	Virus	Normal	Virus	Normal	Virus	Normal	Serum controls
DS 12	Convalescent after natural infection (KEN strain)	++++	0	+	0	+	0	++	0	+	0	+	0	+	0	++	0	0
DE 31	Normal young dog, presumably non-immune	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Antigen controls	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.

Table 2. *Complement fixation with methanol-precipitated antigens derived from various virus specimens*

Virus			Source of sera and immune status of the dogs		
Strain	Dog no.	Specimen	DS 12 (convalescent after naturally acquired infection)	DE 31 (young dog presumably non-immune)	Antigen controls
KEN	DE23	Livers	++++	0	0
KEN	DO47	Spleens	+++	0	0
KEN	DO32	Lungs	++	0	0
KEN	DO46	Peritoneal fluid	++++	0	0
		Mesenteric lymph-nodes	++++	0	0
BECK	DE38	Peritoneal fluid	++++	0	0
		Red blood cells	++++	0	0
		Serum	++++	0	0
Serum controls			0	0	.

(7) *Methanol-precipitated antigens prepared from dog liver damaged by causes other than virus hepatitis*

The occurrence of a number of different non-specific serological reactions in human patients with virus hepatitis has been described. The investigations of Eaton, Murphy & Hanford (1944) and Olitzki & Bernkopf (1945) suggested that the production of anti-liver antibodies occurs in some subjects whose livers have been damaged from various causes. A similar suggestion has been made by Miles (1946). In the light of this evidence several experiments were carried out to demonstrate whether or not the positive antigens we have been investigating were specifically related to canine hepatitis virus or to some non-specific products of hepatic injury. In these experiments three series of methanol-precipitated antigens were tested by the complement-fixation test.

The first series of antigens were prepared from the livers obtained from dogs DE 27 and DE 32 which were infected with KEN strain virus. The dogs were killed 6 and 7 days respectively after virus inoculation, and on pathological examination neither necrosis nor inclusion bodies were discovered in the livers but the hepatic cells showed vacuolation of the cytoplasm. Nevertheless, the presence of virus in the livers was confirmed by subsequent dog inoculation (Parry *et al.* 1951). The second series of antigens were prepared from livers harvested from three dogs: CS 86, CS 87 and CS 98, which had had acute necrosis of the liver of unknown aetiology. The third series of antigens were derived from normal dog livers. Table 4 summarizes the results of the complement-fixation test with these antigens against sera either from ten dogs convalescent from virus hepatitis or from ten normal dogs.

(8) *Reactivity of antigens derived from different strains of canine hepatitis virus*

The sera used were from dogs convalescent after experimental inoculation with SYD, KEN, BECK and STOCK strains of canine hepatitis virus and the methanol-precipitated antigens were derived from specimens of KEN, BECK and STOCK strains. The virus specimens were as follows: dog liver (freeze-dried dog liver received from Prof. Rubarth was used as the specimen of STOCK strain), blood or peritoneal fluid. A typical example of these cross-tests is illustrated in Table 5.

A similarity in the behaviour of the different strains of virus towards convalescent and normal dog sera can be seen from Table 5. Whether or not there is any essential immunological distinction between these different strains of the virus is a matter for further study.

(9) *Comparison of complement fixation at* 3 *and* 37° C.

By examining antisera against different dilutions of positive antigen it can easily be demonstrated that complement fixation for 16–18 hr. in a refrigerator at 3° C. followed by 1 hr. at room temperature makes the test more sensitive than when the fixation is carried out in a water-bath at 37° C. for 30 min. This is illustrated by the results given in Table 6, when pooled sera from convalescent dogs (infected with KEN strain) were examined against methanol-precipitated antigen derived from dog peritoneal fluid (KEN strain). Similar observations were

Table 3. *Complement fixation with unheated and heated pooled methanol precipitated antigens, series* 107 *and* 108

Sera	Antigen: Treatment	Dilutions: 1/10	1/20	1/40	1/80	Serum controls
Pooled sera from convalescent dogs: DE25, DE26, DE33 (after experimental inoculation with KEN strain)	Unheated	++++	++++	+++	++	0
	Heated at 56° C. for 30 min.	++++	++++	+++	+	.
	Heated at 56° C. for 1 hr.	++++	++++	++±	+	.
	Heated at 100° C. for 1 hr.	+	+	±	0	.
Pooled sera from presumably non-immune normal young dogs: DE34, DE35, DE36, DE37, DE38	All antigens as above	0	0	0	0	0
Antigen controls		0	0	0	0	.

Table 4. *Antigens from virus liver, from dog liver with necrosis or unknown aetiology and from normal dog liver against dog convalescent and normal sera in the complement-fixation test*

Liver antigen origin: Dog no.	Data	Convalescent sera: Total no.	No. with +++ or ++++	No. with + or ++	No. negative	Normal sera: Total no.	No. with +++ or ++++	No. with + or ++	No. negative	Antigen controls
DE27	Killed after experimental infection with KEN strain	10	9	1	0	10	0	0	10	0
DE32		10	9	1	0	10	0	0	10	0
CS86	Died with acute liver necrosis of unknown aetiology	10	0	0	10	10	0	0	10	0
CS87		10	0	0	10	10	0	0	10	0
CS98		10	0	0	10	10	0	0	10	0
	Normal dog liver	10	0	0	10	10	0	0	10	0
	Sera controls	10	0	0	10	10	0	0	10	.

Table 5. *Dog sera against methanol-precipitated antigens derived from four strains of canine hepatitis virus in the complement-fixation test*

Source of sera		Strains of virus			
Dog no.	Immune status	KEN	STOCK	BECK	Serum controls
DE 33	Convalescent after experimental inoculation with KEN strain	++++	++++	+++	0
DE 34		+++	++++	+++	0
DO 59	Convalescent after naturally acquired infection with KEN strain	+++	++++	+++	
DO 43		+++	++++	+++	0
DO 36		++	+++	+++	0
DL 9		+++	++++	++++	0
DE 42	Convalescent after experimental inoculation with BECK strain	+++	Not tested	++++	0
DE 62	Convalescent after experimental inoculation with SYD strain	++++	+++	++++	0
DE 64		++++	++++	++++	0
DE 65	Convalescent after experimental inoculation with STOCK strain	++	Not tested	+++	0
DE 67		++	Not tested	+++	0
DE 35	Normal young dogs, presumably non-immune (pre-inoculation period)	0	0	0	0
DE 36		0	0	0	0
DE 37		0	0	0	0
DE 38		0	0	0	0
DE 39		0	0	0	0
DE 40		0	Not tested	0	0
DE 41		0	0	0	0
	Antigen controls	0	0	0	.

Table 6. *Convalescent pooled serum (KEN strain) tested by the complement-fixation test at 3 and 37° C. against methanol-precipitated antigen (KEN strain)*

Fixation temperature (° C.)	Complement: m.h.d.	Complement: Control for stability		Antigen dilutions 1/10	1/20	1/40	1/80	Serum control
37	2	—	Convalescent serum	+	+	±	0	0
			Normal serum	0	0	0	0	0
			Antigen control	0	0	0	0	.
3	2	0	Convalescent serum	++++	++++	+++	+++	+++
			Normal serum	++++	++++	+++	+++	+++
			Antigen control	++	+	±	0	.
	3	0	Convalescent serum	++++	+++	++	±	0
			Normal serum	0	0	0	0	0
			Antigen control	0	0	0	0	.
	4	0	Convalescent serum	+++	+	+	0	0
			Normal serum	0	0	0	0	0
			Antigen control	0	0	0	0	.

Table 7. *Convalescent dog sera (KEN strain) against methanol-precipitated antigen (BECK strain), tested by the complement-fixation test at 3 and 37° C.*

Fixation temperature (° C.)	Complement: m.h.d.	Complement: Control for stability	Convalescent dog sera: DE34 Main tube	DE34 Control	DE48 Main tube	DE48 Control	DE49 Main tube	DE49 Control	DE44 Main tube	DE44 Control	Normal serum (pool) Main tube	Normal serum (pool) Control
37	2	.	++	0	+++	0	+++	0	+	0	0	0
3	2	0	++++	+++	++++	++++	++++	++++	++++	++++	+++	+++
	3	0	+++	0	++++	0	++++	0	++++	0	0	0
	4	0	+++	0	+++	0	+++	0	+++	0	0	0

also made with individual sera from some other convalescent dogs (inoculated with KEN strain) tested against methanol-precipitated antigen derived from dog peritoneal fluid (BECK strain). These experiments are shown in Table 7.

(10) *Comparison of antigens derived from canine hepatitis virus with those from canine para-distemper virus*

These experiments were carried out to demonstrate whether or not there is any similarity between antigens derived from two apparently entirely different viruses. Three ferret spleens, PD 2, PD 3 and PD 4, were used as para-distemper virus-rich material (KENT strain) for the preparation of methanol-precipitated antigens. Canine hepatitis virus antigens were prepared from virus-rich peritoneal fluid (KEN and BECK strains). These antigens were tested by the complement-fixation test against sera from convalescent young dogs which had been experimentally inoculated with KEN strain canine hepatitis virus, but had previously had neither para-distemper nor distemper infections.

No complement fixation occurred with para-distemper virus antigens, while all sera tested gave positive reactions (++, +++ and ++++) with antigens prepared from hepatitis virus.

B. *Serological investigations using the precipitin reaction*

(1) *Comparison between the results of the ring-precipitin and complement-fixation tests*

The purpose of this set of experiments was to determine whether dog convalescent and hyperimmune sera, which had given positive results in the complement-fixation test, would also give a positive precipitin reaction. Forty dog sera positive in the complement-fixation test were examined, at least twice, with the ring-precipitin test against unpurified and methanol-precipitated antigens derived from virus-rich specimens of KEN and BECK strains. The results were in accordance with those obtained in the complement-fixation test. Normal dog sera and antigens negative by the complement-fixation test, which were used as controls, were negative in the precipitin test.

It seemed that precipitins and complement-fixing antibodies appeared in convalescent dog blood approximately at the same time. Table 8 records a sample protocol showing the time of detection of both antibodies in convalescent dog sera using two pooled methanol precipitated antigens (nos. 107 and 108) derived from peritoneal fluids of dogs DE47 and DE38 (KEN and BECK strains respectively).

Rubarth (1947) detected complement-fixing antibodies in sera of experimental dogs on the 13th and 24th days after experimental inoculation and on the 11th and 20th days after the manifestation of the infection. Table 8 shows that the data obtained on the detection of precipitins and complement-fixing antibodies are in accordance with those of Rubarth, although it seemed that the time of appearance of the antibodies might vary.

Incubation temperatures of 37 and 45° C. gave the same results in the ring-precipitin test, nor did the previous inactivation of test sera at 56° C. for 30 min.

influence the results. The optimal time of incubation was established as 2 hr. and the positive reaction became less pronounced if the test-tubes were incubated longer or if they were kept after incubation overnight in the cold.

After the antigens had been heated at 56° C. for 1 hr. or at 100° C. for 30 min., they still gave positive ring-tests with the antisera used.

Table 8. *Time of detection of precipitin and complement-fixing antibodies in dog convalescent sera*

Dog no.	Source of sera	Days since experimental inoculation	Days since manifestation of the infection
DL9	Natural infection (KEN strain)	—	14
DL10		—	14
DE33	Experimental inoculation with KEN strain	51	12
DE34		21	14
DE45		23	21
DE48		23	21
DE62	Experimental inoculation with SYD strain	21	18
DE64		22	18
DE65	Experimental inoculation with STOCK strain	42	38

(2) *Comparative investigations of methanol-precipitated antigens by quantitative complement-fixation and flocculation tests with antisera*

Methanol-precipitated antigen (pooled series nos. 107 and 108) was used in these experiments against non-inactivated dog convalescent and hyperimmune sera.

In contrast to the ring-precipitin test a far longer time of incubation was necessary to carry out the quantitative flocculation test. It was also established that the reaction failed to appear in antigen-antisera mixtures if they were maintained at 45° C. even for 24 hr. At the same time, distinct turbidity appeared in the mixtures which were kept at 37° C. and it seemed that the optimal time for this exposure was 16–18 hr. The fluids in the control tubes with antigen or antisera alone, as well as with antigen-normal sera mixtures, were only opalescent. Therefore, all further experiments with the flocculation test were conducted at 37° C. and the reaction readings were carried out after 16–18 hr. comparing all antigen-antisera mixtures with their respective controls.

The very first comparative experiments showed that the same methanol-precipitated antigen had been more active in the quantitative flocculation test than in the quantitative complement-fixation test. This is shown in Table 9, which illustrates a sample protocol of the antigen examination in both tests against DO 56 dog convalescent serum.

Similar results were obtained with dog hyperimmune sera. As can be seen from Table 10, the quantitative fixation test showed that these sera had slightly different titres which were perhaps due not so much to the different methods of hyperimmunizing the dogs as to their individual abilities as antibody producers.

Typical results of the titration of the methanol-precipitated antigen against pooled hyperimmune sera in the quantitative flocculation test are shown in the sample protocol in Table 11.

Table 9. *Comparative titres of the methanol-precipitated antigen in the quantitative complement-fixation and flocculation tests*

	Serum dilutions						
Antigen titre	1/5	1/10	1/20	1/40	1/80	1/160	1/320
In the complement-fixation test	1/10	0	0	0	Not tested		
In the flocculation test	1/160						

Table 10. *Hyperimmunized serum titres in the quantitative complement-fixation test*

Serum no.	Method of hyperimmunization	Antigen dilutions 1/10	1/20	1/40	1/80	1/160
DS 12	Intravenous	1/20	1/10	1/5	0	0
DE25	Intravenous	1/40	1/20	1/5	1/5	0
DE33	Subcutaneous	1/40	1/20	1/10	1/10	0
DE26	Intraperitoneal	1/20	1/10	1/10	1/10	0

Table 11. *Methanol precipitated antigen against hyperimmune serum (pool) in the quantitative flocculation test*

Serum dilutions ...	1/10	1/20	1/40	1/80	1/160	1/320
Antigen titre	1/5120				1/2560	1/160

IV. DISCUSSION

These experiments leave little doubt that the various body tissues and fluids from dogs infected with hepatitis virus contain antigen specifically related to the virus itself. The nature of the thermostable reactive substance is not yet clear, although solubility studies suggest that lipid fractions are probably associated with the antigenic complex. It is also reasonable to assume that the methanol-precipitated reactive substance is biochemically different from the alcohol-soluble non-specific antigens from the liver described by Eaton *et al.* (1944) and later by Olitzki & Bernkopf (1945).

A comparison of antigens, derived from various virus-rich tissues and fluids, failed to reveal any significant difference in their specificity. Indeed their identity seems to be established and antigens from various sources of virus-rich material can be used interchangeably. On the other hand, the experiments with antigens from canine hepatitis and para-distemper viruses stress the fact that the first antigens have a definite immunological specificity.

Specific antibodies for canine hepatitis virus can be detected by both complement-fixation and precipitin tests; thus the virus is capable of producing precipitins and complement-fixing antibodies in infected dogs and can be studied by these tests in much the same way as other viruses.

The cross-testing of antigens and sera from different strains of virus suggests that they are antigenically identical, but minor antigenic differences might not have been revealed by the experiments described in this paper.

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The preparation of purified antigen by methanol precipitation of various virus-rich tissues and fluids from cases of canine hepatitis which is capable of fixing haemolytic complement and is detectable by the precipitin test is described in this paper. The term 'purified antigen' as used here refers to preparations from which a large proportion of host protein has been removed, but this is not intended to suggest that the antigenic substance has been obtained completely free from impurities. The study of the antigen here described is not meant to be a final one. There is still much to learn about the nature of the antigenic complex as well as about its standardization, stability on storage, inactivation, etc. Meanwhile, the methanol-precipitated antigens used with the serological tests described have given constant and clear-cut results in many experiments, and certainly afford a basis for further work.

Complement fixation at 3° C. is definitely more sensitive than fixation at 37° C. At the same time, fixation in the cold is a more elaborate method, and much higher doses of complement are necessary to carry it out; this is not always desirable. Hence both methods are valuable and should be used in conjunction with each other during investigations of complement-fixing antigens or antibodies related to canine hepatitis virus.

The relationship between these complement fixing and precipitating systems and the possibility of protection against infection by the injection of antigen or neutralizing antibodies remains to be studied. The methods of preparation and testing of antigens derived from infected liver may well prove of service in later studies of the inter-relationship between canine hepatitis and other examples of mammalian virus hepatitis, including hepatitis of man. They may also prove useful in the study of methods of producing artificial active immunity.

V. SUMMARY

The preparation of purified antigen from canine hepatitis virus sources is described. This includes the manufacture of crude antigenic suspensions and their subsequent treatment to remove non-specific interfering substances.

The solubility of the antigenic substance in various solvents has been studied and its stability is briefly discussed.

The complement-fixation and precipitin tests are described in detail as they have been used for the virus studies.

Various virus-rich body tissues and fluids have been serologically tested, and the identity of antigens prepared from them as well as the antigenic similarity of the various strains of canine hepatitis virus have been established.

Evidence has been recorded that canine hepatitis virus produces serologically distinct antibodies, as measured by the precipitin and complement-fixation reactions in the sera of dogs during convalescence. The titres of precipitating and complement-fixing antibodies in hyperimmune dog sera appeared to run parallel to one another.

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