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

III. THE PROPERTIES OF THE COMPLEMENT-FIXING ANTIGEN AND ITS PROBABLE STRUCTURE

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(With Plates 1–3 and 1 Figure in the Text)

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INTRODUCTION

Canine hepatitis virus is notable for specific and highly active complement-fixing antigen which appears in tissues and body fluids of dogs affected with the disease under natural or experimental conditions. In the preceding papers (Larin, 1951 *a*, *b*) a method was described whereby the virus antigen could be purified from the bulk of proteins in tissue suspensions or body fluids. This involved a chemical precipitation with methanol in the cold, and subsequent elution of the active material from the precipitate with either distilled water or saline. The antigenic activity was found to be associated with material which remained in the supernatant after high-speed centrifugation for 30 min. at 25,000 *g*.

Since the purified material was antigenically active *in vitro* and also infective *in vivo* (Larin, 1951 *b*), it seemed important to investigate the nature and properties of the antigen, as such a study might assist in further studies of the nature of the virus particle and its life cycle. The present paper describes experimental data bearing on the chemical properties and probable structure of the antigenic material.

MATERIALS AND METHODS

Most of the experimental techniques and materials used have been described in detail in the preceding papers (Larin, 1951 *a*, *b*).

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Virus strains: BECK, KEN and STOCK strains (Larin, 1951*a*) were used in all experiments.

Isolation of liver cell nuclei: the methods described by Dounce (1943*a*, *b*) and Vendrely (1952) for the isolation of cell nuclei were used. Both methods have given good preparations of isolated nuclei, suitable for a variety of studies.

High-speed centrifugation: this was carried out in the angle head of a M.S.E. Major centrifuge with cooling unit.

Wherever other materials or techniques have been used they are described below.

COMPLEMENT-FIXATION TEST

The method of performing the test was fundamentally that described in previous papers (Larin 1951*a*, *b*), fixation at 37° C. for 30 min. being used.

The standard diluent Ca–Mg saline, buffered with veronal, was prepared according to Wallace, Osler & Mayer (1950). The autoclaved sterile solution had a pH of 7·25–7·30.

Specific antisera were obtained from dogs hyperimmunized with the virus, one antiserum pool being used throughout all the experiments. On the day of the test the standard antiserum was diluted 1/5 and inactivated by heating for 30 min. at 56° C.

Pools of guinea-pig serum were prepared and used fresh or frozen and stored in sealed glass ampoules on dry ice. Guinea-pig complement was always titrated and standardized for each experiment, and each experiment was controlled by titrations of complement plus antigen and complement plus serum.

The haemolytic indicator consisted of 3 % sheep red cells sensitized with 5 units of commercial haemolytic amboceptor (Messrs Burroughs, Wellcome and Co.). Each preparation of the antigen was tested for anticomplementary, lytic, non-specific and specific complement-fixing properties. The strength of the virus antigen was determined by serial dilution tests with a constant amount of antiserum and in the presence of different quantities of complement for each dilution of the antigen, the dilution of antigen showing 50 % haemolysis being taken as the end-point. Values representing 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 % lysis were read by eye with the use of standards. The values of K', the amount of complement required for 50 % haemolysis, were estimated for each dilution of antigen, and the complement-fixing capacity, in units per ml., was determined by extrapolation. A preparation of the antigen is said to have a titre of x units when a dilution of $1/x$ in 0·5 ml. volume used in the complement-fixation technique described (total volume in the test being 2·0 ml.) gives 50 % haemolysis. A high degree of reproducibility and accuracy was maintained throughout all the experiments, the standard deviation of replicate results on different days of testing being under 1 %.

A typical protocol of antigen titrations is recorded in Text-fig. 1 which shows complement-fixing activity of ten stock virus antigens each in the dilutions of 1/25. Considered as a whole Text-fig. 1 shows that virus and control antigens were practically not anticomplementary, their haemolytic properties being nil. Anticomplementary properties of the standard antiserum (1/5 dilution), though more marked than those of the antigens, did not interfere with the specific fixation of complement by the antigen-antibody union.

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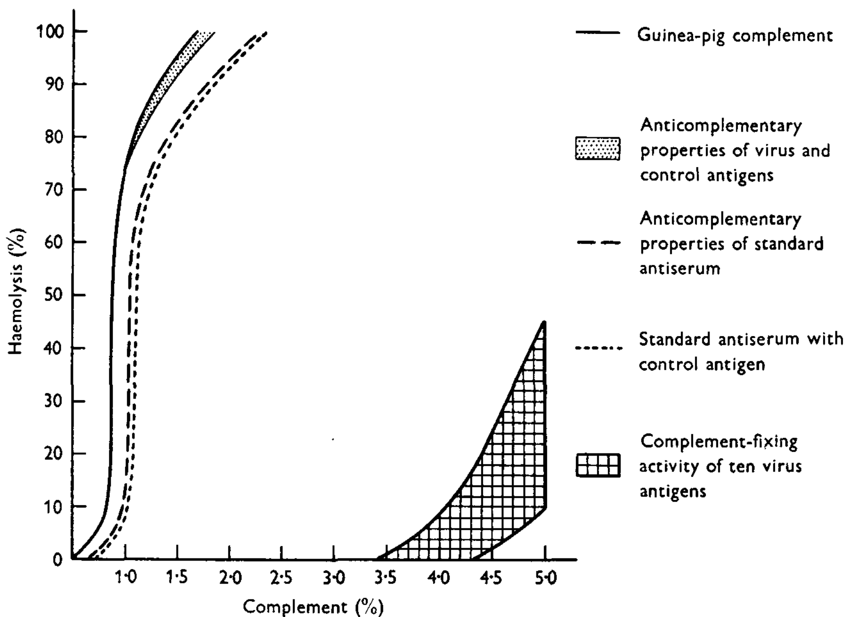
BIOLOGICAL PROPERTIES OF THE VIRUS TISSUE SUSPENSION

The suspension was infective and contained the specific complement-fixing antigen. These properties were completely removed from it by the addition, in the cold, of methanol up to 40 % of its volume. Methanol produced a precipitate from which both the infective virus and the complement-fixing antigen could be recovered by elution with distilled water or saline. Since the blood of moribund dogs was infective and antigenically active *in vitro* the distribution of virus and antigenic material in the body was probably governed by the distribution of blood, and hence it was difficult to judge the amount of the virus and antigen in other tissues or body fluids.



Text-fig. 1. Complement-fixing activity of ten virus antigens (1:25 dilution) with standard antiserum (1:5 dilution) in the presence of varying amounts of complement.

The presence of a high concentration of the complement-fixing antigen in tissues and body fluids always coincided with the presence in the virus lesions of the intranuclear inclusion bodies.

No intranuclear inclusion bodies were found in tissues of fifteen experimental dogs killed 3 months after complete clinical recovery from the usual 7–10 days course of the acute disease. However, the parenchymal cellular damage with intralobular and peripheral loss of cells, wide distension of sinusoids, and thinning of the cords of the liver cells, very closely resembled the characteristic histopathological features of the acute form of the disease. Since the recovered dogs did not show any sign of a relapse of the disease, it remains a serious possibility that the clinical signs of canine virus hepatitis are not due to liver damage alone. This assumption is supported by pathological findings in fatal cases which show that

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the vascular system as well as other organs of vital importance are damaged by the virus no less than the liver. Liver suspensions from these dogs were infective, but their antigenic activity *in vitro* was low.

As with human virus hepatitis, in the canine disease residual tissue damage can persist for a long time after clinical recovery, in some dogs for more than 1 year. In fact, our observations made on a few hundred dogs during 4 years showed that a proportion of cases probably never fully recover, and may die of another infection however benign that may be (Larin, 1953; Laughton & Larin, 1954).

It can be seen from Pl. 1 that the pattern of residual damage in the liver of dogs 15 months after complete clinical recovery from the disease resembled the characteristic histopathological features of the acute disease (Pl. 2) and also that necrotic changes in residual liver lesions (Pl. 3, fig. 1) were well marked, showing a considerable delay in the natural healing process. Nevertheless, the intranuclear inclusion bodies which are characteristic of the fatal disease (Pl. 2 and Pl. 3, fig. 2) were never seen in the residual lesions (Pl. 1 and Pl. 3, fig. 1), which contained so little complement-fixing antigen that its presence was not proven. On the other hand, the residual lesions still contained infective virus which was recovered by inoculation of the tissue suspension into susceptible dogs. These cases are interpreted as recrudescences of infection in a subclinical form with a resulting delay in the natural healing process.

It is of interest to mention here that the intranuclear inclusion bodies were not detectable in tissues of chick embryos inoculated with the virus; neither did the embryos contain the antigen in detectable amounts, although they produced the characteristic disease in inoculated dogs. Moreover, intranuclear inclusion bodies were not detectable in tissues in most suckling puppies dying of the acute disease under natural or experimental conditions. Inclusion bodies were observed in only one case of virus hepatitis in suckling puppies out of many hundreds examined. A second case of the disease with the inclusion bodies present in a week-old puppy has been reported by Skulski (1955, personal communication). Tissues of suckling puppies dying of the disease contained infective virus, but the complement-fixing antigen was either present in low concentration or was not proven.

The antigenic activities *in vitro* of virus suspensions prepared from the liver tissues of suckling puppies and from young and adult dogs, all dying from the acute disease, are compared in Table 1.

The data in Table 1 illustrate the point that, although the virus can proliferate with no intranuclear inclusion bodies detectable, the complement-fixing activity of the virus is in some way associated with their presence.

EXPERIMENTS WITH ISOLATED NUCLEI CONTAINING THE INCLUSION BODIES

Recent studies of the antigen of canine hepatitis virus by means of fluorescent antibody (Coffin, Coons & Cabasso, 1953) provided evidence that the accumulation of specific antigenic material begins on the nuclear membrane, and that the

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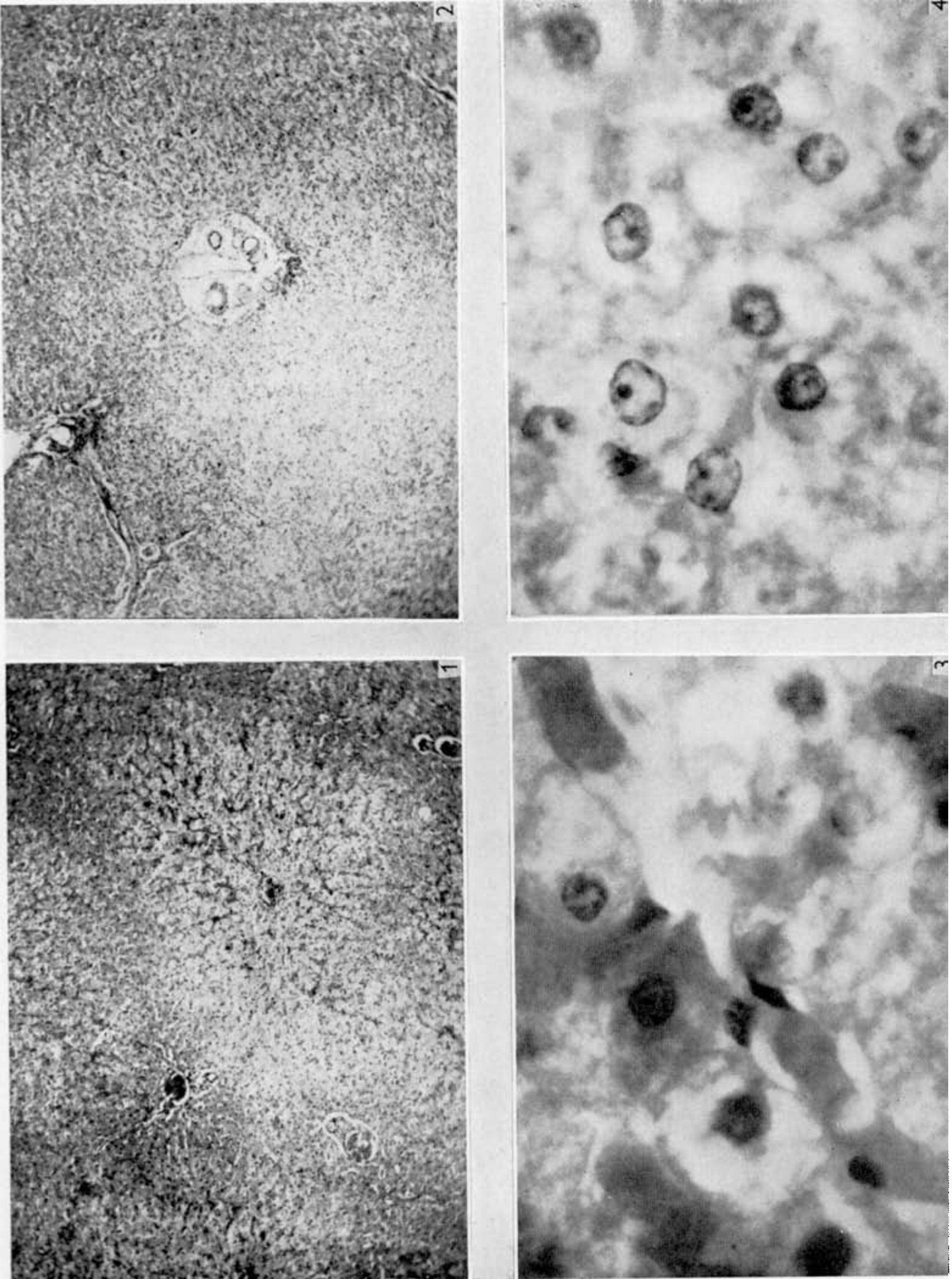

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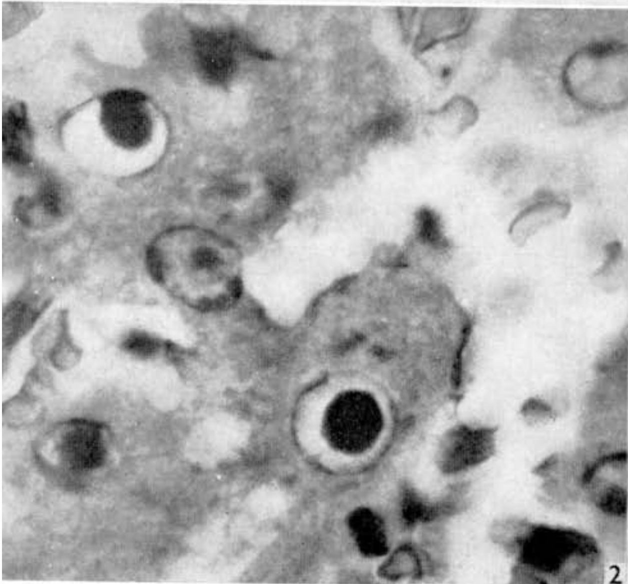

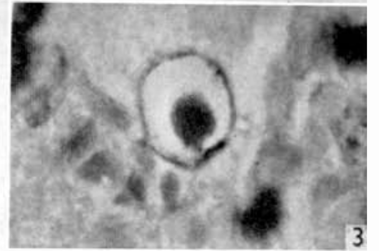

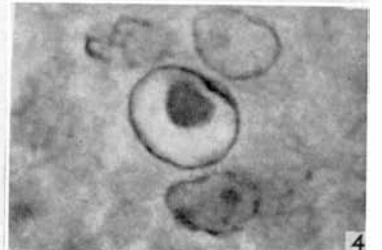

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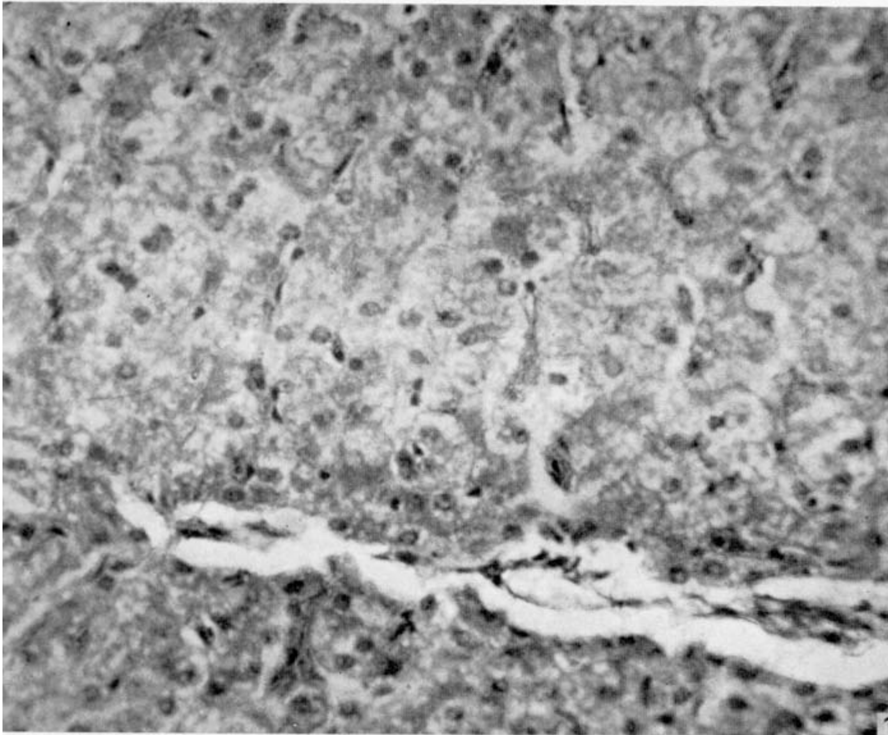

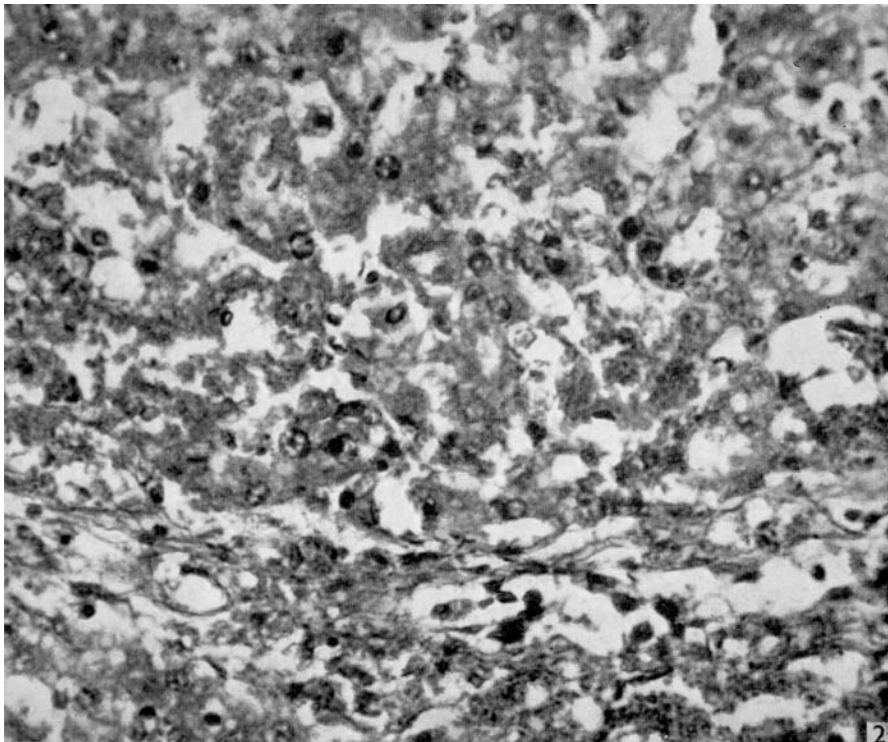

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peripheral area is probably composed of active virus particles. These data and our own observations suggested that the inclusions might be collections of antigen. To test this, the nuclei of liver cells containing inclusion bodies were isolated from the cytoplasm by the use of one of the methods referred to above. Complement-fixation tests showed that the antigen was detectable in cytoplasmic fractions but not in the isolated nuclei. From these experiments it follows that whatever the relationship between the antigen and the inclusion bodies may be, the latter are not just depots of the material antigenically active *in vitro*.

Table 1. *Titres of complement-fixing antigen in the liver tissue of suckling puppies compared with the titres in adult dogs, all dying from the disease*

Titre of antigen in C.F. units (per 1 g. wet liver tissue)	Percentage of suckling puppies	Percentage of young and adult dogs
0	60	—
50	23	—
100	10	—
200	5	—
500	2*	—
1,000	—	—
2,000	—	45
4,000	—	30
8,000	—	20
16,000	—	5

* The intranuclear inclusion bodies were present in both of these puppies.

THE EFFECT OF METHANOL

Because methanol seems to denature and precipitate somatic proteins without damaging virus which is precipitated together with the proteins and which can be subsequently eluted with a solvent from the precipitate, it offers a means of purifying and concentrating the virus and antigen (Larin, 1951*a*, *b*). The optimum concentration of methanol was determined by adding 5, 10, 20, 30, 40, 50 and 60 % of cooled methanol to aliquots of 10 % virus tissue suspension. By testing the resulting antigen eluted in M/15-phosphate buffer pH 7·0, it was found that concentrations of methanol from 5 to 30 % failed to precipitate antigen completely, 40 % methanol gave complete precipitation and stronger concentrations destroyed the antigen. Further experiments showed that the antigen could be easily eluted with any of the M/15-phosphate buffers at pH ranging from 5·0 to 9·0 or with distilled water or saline, all the eluates being similarly active.

Centrifugation at 25,000 *g* for 30 min. reduced the solid content of the eluates but not at the expense of the antigen. Centrifuged antigen eluates made with double distilled water contained on the average 9·7 mg. of solids (dry weight) per 1 ml. of the fluid containing 800 complement-fixing units of antigen.

Attempts to concentrate the antigen by using small volumes of the elution fluids and also to obtain purer preparations by precipitation of the antigen in the

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eluates with methanol were unsuccessful because of loss of complement-fixing power.

From the above results it followed that, while the antigen in tissue suspensions and body fluids was not denatured by methanol, all attempts to precipitate purified antigen from the eluates resulted in a loss of complement-fixing powers despite careful control of methanol concentration and temperature of the reaction. It seemed as if either the purification procedures had modified the antigenic material in the eluates and made it more sensitive to methanol, or probably the antigen in tissue suspensions and body fluids was protected from the action of methanol by some substances which were removed during the purification. An accidental observation that the antigen was completely lost from a sample of the virus peritoneal effusion during the routine purification procedures made it necessary to examine the methanol precipitation technique in relation to yields of active material in the eluates. In these experiments twenty-five samples of the virus peritoneal effusion, each derived from a different dog dying of the disease, were used for purification of the antigen by the methanol precipitation technique. Before the purification the antigen was present in all twenty-five samples of the peritoneal effusion. After the methanol precipitation the antigen was completely eluted from the precipitates in twenty-two samples; from the precipitate in one sample about half of the antigenic material only could be eluted and no antigen was recovered from the precipitates in two samples. Several attempts to purify the antigenic material from aliquots of the latter two samples with methanol added in concentration from 5 to 30 % have all resulted in the loss of the antigen.

These experiments, taken in conjunction with the failure to precipitate purified antigen in the eluates, suggest that whatever may be the nature of the antigen inactivation in the two samples of peritoneal effusion, this indicated that the sensitivity to methanol of purified antigen in the eluates was not due to a modification of the antigenic particle caused by the purification procedures.

DIGESTION WITH PROTEOLYTIC ENZYMES

The antigenic material was not completely destroyed by digestion with trypsin or chymotrypsin. Preparations of the purified antigen were digested at pH 8·5 overnight at 37° C. with trypsin or crystalline chymotrypsin in concentrations of 1/1000, 1/2000 or 1/20,000 with the results shown in Table 2.

Table 2

Enzymes	Antigen titre in complement-fixing units
Antigen control (no enzymes added)	500
Trypsin 1/1000	100
Trypsin 1/2000	200
Trypsin 1/20,000	500
Chymotrypsin 1/1000	300
Chymotrypsin 1/2000	400
Chymotrypsin 1/20,000	500

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Solutions of the enzymes tested as controls in the complement-fixation reaction showed no anticomplementary properties. Neither of these solutions produced non-specific complement fixation with either the antigen or antiserum, nor did they cause lysis of the haemolytic indicator.

It is apparent from Table 2 that, although the antigen was obviously digested by trypsin and chymotrypsin, the amount of enzyme was large, and total destruction of the antigen was not achieved.

PRECIPITATION WITH AMMONIUM SULPHATE

Purified antigenic material in distilled water was precipitated by half-saturation of the fluid with ammonium sulphate, and the antigen was recovered by dissolving the precipitate in distilled water. These procedures have, however, always been associated with a loss of complement-fixing powers.

PRECIPITATION WITH LANTHANUM ACETATE

Nucleoproteins form insoluble complexes with salts of metals of the alkaline earth group by combination with the nucleic acid. In order to ascertain whether the antigenically active material was composed of nucleoprotein, preparations of purified antigen were made from the virus tissue suspensions and virus peritoneal effusion, the bulk of inactive proteins having been removed by methanol precipitation. From these purified preparations, and also from the original virus tissue suspensions and peritoneal effusion, the antigen was completely precipitated by the addition of 1 % lanthanum acetate in volumes equal to those of the antigenic aliquots; the supernatant fluid, after removal of excess lanthanum by precipitation with 2·5 % Na_2HPO_4, having no complement-fixing power.

This precipitation of the antigenic material was obviously not a simple salt precipitation, since the antigen, which is normally soluble in distilled water, could not be recovered from the precipitate by repeated washing with distilled water. However, the antigen could be eluted from the precipitate by washing with 2·5 % Na_2HPO_4. It follows from these experiments that the antigen probably contains nucleic acid, and, thus, it is possible that the antigenic particle is composed of nucleoprotein.

THE EFFECT OF ETHANOL

The antigenic material in virus tissue suspensions or body fluids can be precipitated by ethanol, but the eluates appear to be less active than those obtained with methanol (Larin, 1951*a*).

As ethanol denatures proteins but leaves the nucleic acid, experiments were made to ascertain whether the nucleic acid of the antigen was serologically active. In these experiments the antigenic material in aliquots of the virus tissue suspension was completely precipitated, at room temperature, by treatment with absolute ethanol added in volumes equal to those of the aliquots. The precipitate was largely insoluble, and the antigenically active material could be detected in neither the

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supernatant, after ethanol had been evaporated *in vacuo*, nor the precipitate. These results suggest that the nucleic acid of the antigen was not serologically active.

FILTRATION EXPERIMENTS

The antigen prepared from all sources, and in all these experiments, was strongly adsorbed onto Seitz clarifying (F.C.B.) disks and by filter paper. There was no noticeable adsorption of the antigen onto Doulton kieselguhr filter candles (KS) with maximum pore diameters from 2·0 to 2·5μ.

ESTIMATION OF THE ANTIGEN PARTICLE SIZE BY ULTRAFILTRATION

The value of ultrafiltration as a method for measuring the size of viruses was critized by Levaditi, Paic & Krassnoff (1936), who drew attention to certain pitfalls in the use of ultrafiltration methods. Similar criticism can be made in connexion with the ultrafiltration of the antigen because the filtrates were tested for the presence of the active material by the complement-fixation test, and thus, the factor liable to influence the results was the sensitivity of the antigen-antibody reaction *in vitro*.

On the other hand, since the sensitivity of the complement-fixation reaction does not vary from test to test as much as a virus possibly does in virulence or in pathogenicity for different animal species, it was considered that the ultrafiltration of the antigen should provide valuable data bearing evidence on the particle size of the antigenically active material.

Several batches of virus peritoneal effusion and also of the antigen purified by precipitation either with methanol or with lanthanum acetate were used in these experiments; the antigenic preparations being centrifuged at 25,000 g for 30 min. in order to obtain more purity. Each batch of the antigen used in these experiments was divided into two: the first part was filtered consecutively through Gradocol membranes of pore diameters from 720 to 140 mμ, the second was divided into seven aliquots, corresponding to seven pore diameters used, each being filtered through a Gradocol membrane of one particular pore diameter only. The ultrafiltrations were made under negative pressure. The ultrafiltration experiments were repeated three times, and the results were sufficiently in accord to warrant publication.

In Table 3 data are given representing the mean values of the antigenic activity obtained from the above experiments.

From the data in Table 3 it appears that Gradocol membranes of average pore diameter 530 mμ retained about 36 % of the active material. Gradocol membranes of 320 mμ retained 60 %, and membranes of 210 mμ pore diameter retained about 90 % of the antigen. The filtrates through membranes of 140 mμ pore diameter were either antigenically inactive, or the titres were so low that the presence of the antigen was not proven.

In this series of experiments a very interesting situation arose. This was the failure of all attempts to recover the active material retained by the Gradocol

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membranes, although the membranes were thoroughly minced and repeatedly extracted with a suitable solvent.

From the results of the ultrafiltration experiment it is clear that the antigenic material definitely consists of organized particles. Secondly, by filtering the antigen through Gradocol membranes of graded porosity it was shown that the antigenic particle was completely retained by membranes of 140 mμ pore diameter. Hence, one can estimate by using Elford's correction factor (Elford, 1933) that the diameter of the active particle is probably between 70 and 105m μ.

Table 3. *Titre of the antigen in Gradocol filtrates compared with non-filtered antigen*

Pore diameter (in mμ)	Titre in C.F. units of the antigen purified by		
	Unpurified	Methanol precipitation	Lanthanum acetate precipitation
Unfiltered antigen (control)	500	500	500
780	500	500	500
620	500	500	500
530	310	320	330
430	280	270	280
320	200	200	210
210	50	50	45
140	0	0	0

DISCUSSION

The experiments described in this paper enable us to formulate a fairly complete picture of the properties and probable structure of the complement-fixing antigen, although this picture may require some modification of its detail as a result of further work.

It can be considered as established that the complement-fixing activity of canine hepatitis virus is due to 'particulate' antigen which was demonstrated in the preparations of unpurified antigen and also in preparations of antigen purified by either methanol or lanthanum acetate precipitation. The chemical properties of the antigen are those of protein, probably ribonucleoprotein. It is obvious from the foregoing account that the size of the active particle is probably between 70 and 105 mμ.

The results of the ultrafiltration in Table 3 show that membranes of 210 mμ retained about 90 % and membranes of 140 mμ 100 % of the antigen which could not be recovered, even though the membranes, after being thoroughly minced, were repeatedly extracted with a suitable solvent. The interpretation of these results is difficult because it is not possible to be absolutely certain that the antigen was not adsorbed on to the filter. On the other hand, the data in Table 2 show that there was no noticeable adsorption of the antigen on to the membranes of 620 and 780 mμ porosity diameter. This evidence, taken together with the strong adsorption of the antigen on to Seitz disks, lends weight to the suggestion that a progressive clogging of the pores during the ultrafiltration of the antigen through Gradocol membranes probably caused aggregation of the retained particles.

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These experiments provided no evidence on the association of the presence of the antigen in a high concentration with the appearance of numerous intranuclear inclusion bodies in virus lesions. Since the inclusion bodies appear only in the fatal form of the disease, and only in young and adult dogs, not in suckling puppies or chick embryos inoculated with the virus, it is possible that the formation of the inclusion bodies depends rather upon the virus life cycle in the body than upon the vigour of virus attack on the cell. Since the intranuclear inclusion bodies (Pl. 2, figs. 2–4 and Pl. 3, fig. 2), and also the complement-fixing antigen, are probably composed of ribonucleoprotein, it is possible that they both represent certain stages in the formation of virus protein by a catalytic reaction from the denatured host proteinogen. This process will remain obscure until the virus life cycle is investigated stage by stage.

SUMMARY

The complement-fixing antigen activity of canine hepatitis virus is due to particles which are estimated to measure from 70 to 105 mμ and which probably can form larger aggregates. The active particles in the original virus tissue suspension were of sizes similar to those in the purified preparations, their serological properties being identical. The chemical properties of the antigen are those of protein, probably ribonucleoprotein. No evidence has been obtained to explain the association of a high concentration of the antigen with the presence of numerous intranuclear inclusion bodies in the lesions. It is evident, however, from these experiments that the inclusion bodies are not just deposits of the serologically active material. Since the intranuclear inclusion bodies and also the antigen are probably composed of ribonucleoprotein, it is possible that they both represent certain stages in the formation of virus protein by a catalytic reaction from the denatured host proteinogen.

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EXPLANATION OF PLATES

Plate 1

Residual liver damage in dogs 15 months after complete clinical recovery from canine virus hepatitis (post-mortem time, 15 min.).

Fig. 1. Autopsy section DO 29. Well marked parenchymal damage with loss of cells. H and E. × 60.

Fig. 3. Same section. The field shows necrotic cells. There are no intranuclear inclusion bodies. × 1200.

Figs. 2 and 4. Autopsy sections DO 56. (× 60 and × 1200). H and E.

Plate 2

Fatal case of acute canine virus hepatitis. Dog DE 52 (post-mortem time, 1 hr.).

Fig. 1. Liver autopsy section. Well marked loss of cells. H and E. × 100.

Fig. 2. Same section as Fig. 1. The field shows necrotic cells and acidophilic intranuclear inclusion bodies. × 1500.

Figs. 3 and 4. Liver autopsy sections. The intranuclear inclusion bodies gave positive reactions to tests for ribonucleic acid. (Fig. 3, Cowdry's Method; Fig. 4, Turchini's Method). × 1500.

Plate 3

Fig. 1. Residual damage 15 months after complete recovery from canine virus hepatitis (post-mortem time, 15 min.). Liver autopsy section. H and E. × 240.

Fig. 2. Fatal case of canine virus hepatitis (post-mortem time, 1 hr.). Liver autopsy section. H and E. × 240.

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