other and from PTH became evident from that fact that the HPU activity in urine was dialyzable while HCE activity was not. Also, HCE activity in urine, on CCD, separated at a point different from both HPU activity and bovine PTH.

Summary. Butanol extracts of normal human and rat urine contained two types of substances, distinct from each other and unrelated to PTH, which interfered with the bio-assay of PTH in urine. One caused hypercalcemia, the other hyperphosphaturia in PTX rats. The latter material was dialyzable and equilibrated between 20% acetic and *n*-butanol with a distribution coefficient (*K*) about 10 times lower than that of PTH. The "hypercalcemic" material was nondialyzable and its *K* about 5 times lower than that of PTH. Thus, both materials could be separated by CCD from PTH added to the urine before extraction. From 100 ml of normal (human or rat) urine, no PTH activity could be detected in fractions expected to contain PTH in its known form.

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Canine Tracheobronchitis: Isolation and Characterization of the Agent with Experimental Reproduction of the Disease (32618)

ABRAHAM KARPAS, NORVAL W. KING, FELIX G. GARCIA, FERNANDO CALVO AND REX E. CROSS (Introduced by J. F. Enders)
The Animal Research Center, Harvard Medical School, Boston 02115

In a preliminary paper (1), the isolation of a herpes virus from a clinical case of infectious canine tracheobronchitis was described. This represented the first confirmed report of herpes virus isolation from natural cases of canine respiratory disease. In the present paper the properties of the virus and preliminary results of experimental infection of dogs with the agent are presented. The virus was found to be identical with herpescanis virus. The pathogenicity of this agent will be evaluated on the basis of our studies and those of previous workers.

Material and Methods. Tissue culture. Monolayer cultures of whole dog embryo, fetal and newborn dog kidney (DK) cells were prepared according to standard techniques and incubated at 37°C. The growth medium consisted of "basal" Eagle's plus 10% calf serum, and the maintenance medium (MM) of Eagle's plus 2% calf serum. The medium contained 200 units/ml of penicillin and 200 μg/ml of streptomycin.

Viruses. The virus to be characterized in this paper was isolated from a dog with a clinical case of tracheobronchitis. It was repassed on DK cell cultures and clarified by centrifugation at 2000 rpm for 20 min. Herpescanis virus for comparative studies was provided by Dr. Carmichael of Cornell University. This virus was handled in a similar manner.

Virus isolations. Three naturally infected dogs with advanced clinical tracheobronchitis were used in the initial study. After taking blood samples from each of them, they were sacrificed and postmortem examination was performed. Trachea and bronchi were washed with Eagle's medium containing 2000 units of penicillin, 2000 μg of streptomycin and 15 μg of Fungizon, per ml. Representative tissue samples of tracheal, laryngeal and bronchial mucosae, retropharyngeal lymph nodes, lungs, livers, kidneys, and spleens were processed for viral isolation. Subsequently nasal and throat swabs were taken from a random group

of 60 dogs from the same kennel, some of which exhibited clinical tracheobronchitis, others of which appeared asymptomatic. All samples were added to cultures of dog cells, and were examined regularly throughout a period of 12 days for the development of cytopathic effects.

Virus titrations. Virus titrations were performed by inoculation of each serial 10-fold dilution of the virus into 3 culture tubes. BME-Earle's Base tissue culture medium (BME)[1] was used as a diluent. End points were determined after incubation for 12 days. The $TCID_{50}$ was calculated according to the method of Reed and Muench.

Susceptibility to physical and chemical agents. The effects of the following agents on the isolated virus were studied using virus stocks with a titer of $10^4 TCID_{50}$ per ml: (a) Thermolability: Virus suspensions were incubated in a water bath at 56°C for 30 min. (b) pH: Virus suspension was adjusted to pH 3.0 and left at room temperature for 30 min. (c) Ether sensitivity tests used were similar to those described by Andrewes and Horstman (2). Virus suspensions were subjected to treatment with 20% (by volume) diethylether for 15–20 hours at 4°C. After the treatments the ether was evaporated.

Treated and control samples were titered on DK monolayers.

Size determination. The approximate size was determined by ultrafiltration with membrane filters (Millipore) of pore diameter 450 mμ, 220 mμ, 100 mμ, and 50 mμ. Aliquots of each sample were passed consecutively through each filter and the remainder was inoculated into DK cells.

Cytology. The DK cells grown on coverslips in Leighton tubes were infected with the isolated virus, and were harvested at different intervals. They were compared with uninoculated control preparations. Each coverslip was washed with BME to remove excess medium, fixed for 1 hour in Bouin's fixative and stained with hematoxylin and eosin (H & E).

Hemagglutination. Agglutination of erythrocytes from rhesus monkey, dog, and rat were tested at pH 6.9. The stock virus was diluted in twofold steps from 1:2 through 1:128 in 0.85% NaCl and 0.5 ml of each virus dilution was added to 0.5 ml of 0.2% suspensions of washed erythrocytes in 0.85% NaCl. Tests were done at 4°C, 22°C and 37°C and were read after 1 and 3 hours of incubation.

Determination of the nucleic acid type. Confluent monolayers of DK cells were washed with BME and inoculated with the viral suspension. Virus was allowed to adsorb at 37°C for 60 min and unadsorbed virus was then removed by repeated washings. The infected cells were divided into three groups: to one was added maintenance medium (MM) alone, to the second MM plus 20 μg of 5-bromo-2-deoxyuridine (BUDR) per ml, to the third 20 μg BUDR/ml + 50 μg thymidine per ml. Five tissue culture bottles were used for each group. Herpessuis (pseudorabies) virus as a control DNA virus (3) was likewise examined in the same cell systems.

On the following day the cultures were examined for CPE. Cultures on the second day were exposed to two cycles of rapid freezing and thawing (—86°C to 35°C), the contents harvested, pooled separately, and centrifuged at 2000 rpm for 20 min. Titrations of the various supernatant fluids were performed as described earlier.

Antisera. Rabbits were given 5 injections of 1 ml each of the virus suspension at 5-day intervals. On the 24th day 5 ml of virus suspension was injected intraperitoneally. The initial bleeding was done 10 days following the last injection.

Susceptible dogs were inoculated intranasally with virus suspensions. The dogs were bled at 3–4-day intervals during the first month and at intervals of 1 month thereafter.

Neutralization tests. Serial twofold dilutions of inactivated serum (56°C for 30 min) were mixed with equal volumes of virus suspension. The viral dose was approximately $10^3 TCD_{50}$/ml. The serum-virus mixture was incubated at 37°C for 1 hour, and then added to DK monolayers. Incubation was continued for 2 hours before the addition of maintenance medium. Presence of neutralizing antibodies was indicated by the absence of cytopathogenic changes. End points were recorded after 1 week of incubation.

Electron microscopy. Cultures showing advanced CPE were frozen and thawed (—86°C

[1] Grand Island Biological Co., Grand Island, N. Y.

TABLE I. The Effect of BUDR on the Multiplication of the Canine Isolate and Herpessuis Virus in the Absence or Presence of Thymidine.

Virus	Infectivity titer (ID_{50}/0.1 ml) of whole culture		
	Maintenance medium (MM)	MM + 20 μg BUDR/ml	MM + 20 μg BUDR/ml + 50 μg thymidine
Canine isolate	10^6	10^2	10^6
Herpessuis	10^7	$10^{4.5}$	10^7

to 35°C) three times, and centrifuged at 2000 rpm for 20 min. The supernatant fluid was then centrifuged in a no. 40 rotor at 30,000 rpm for 1 hour in a Spinco ultracentrifuge (Model L3). The pellet portions were suspended in sterile distilled water and were mixed with equal volumes of 3% phosphotungstic acid (PTA) at pH 6.9. Small drops of the negatively stained virus suspension were dried on carbon coated grids and these were examined in a RCA (model 3H) electron microscope.

Susceptibility of laboratory animals to the virus. Newborn mice, hamsters, and guinea pigs were innoculated with herpescanis virus intracerebrally, intraperitoneally and subcutaneously. Adult rabbits were innoculated intradermally and intravenously. The innoculated animals were kept for several months after inoculation.

Experimental infection of dogs. Seven, 6-month-old dogs, which had been bred by our own kennels and had been previously vaccinated against canine distemper, hepatitis and leptospiroses were utilized. Five of these dogs were inoculated with the virus and were kept under conditions of strict isolation. The other two dogs were used as controls and were kept separately during the first 3 months. Anal, nasal and throat swabs, together with blood samples were taken prior to intranasal inoculation of the virus. After inoculation, the same swabs were collected at intervals indicated in Table II for a period of 3 months. Blood samples were taken at intervals indicated in Table III. The serum was utilized for serological analysis to determine the presence of neutralizing antibodies to the virus.

Several dogs of different ages were inoculated with the virus intranasally and were used for histopathologic studies.

Three, 4-day-old pups were inoculated intraperitoneally with 10^5 $TCID_{50}$ of the virus suspension.

Results. Virus isolations. A virus was isolated from the tracheal and bronchial washings; tracheal, laryngeal, and bronchial

TABLE II. Experimental Inoculation of Dogs with Herpescanis Virus via the Nose.

Dog no.	Prior to inoculation			Days after inoculation													
				3[a]			7			11			14			18		
	A	N	T[b]	A	N	T	A	N	T	A	N	T	A	N	T	A	N	T
11	—	—	—[c]	—	—	—	—	—	—	—	+	+	—	+	+	—	+	+
13	—	—	—	—	—	—	—	+	—	—	+	+	—	+	+	—	+	—
16	—	—	—	—	+	—	+	+	—	—	—	—	—	+	—	—	+	—
20	—	—	—	—	+	+	—	+	+	+	+	—	—	—	—	—	—	—
22	—	—	—	—	+	—	—	+	—	—	+	—	—	—	—	—	—	—
12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
21	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

[a] The numbers along the horizontal axis indicate the days post inoculation when swabs were collected. Dogs #12 and #21 were not inoculated.

[b] A = anal swabs, N = nasal swabs, T = throat swabs.

[c] + = virus recovery, — = no virus recovery.

mucosae; and from the lungs of one of the three naturally infected dogs initially sacrificed for study. No virus was recovered from the other tissues examined from this animal, nor from any of the tissues from the other two dogs. Serologically identical virus was isolated from 10 of the group of 60 dogs from which nasal, throat, and anal swabs were taken. Of this group of 60 animals, only 8 exhibited clinical symptoms but only 3 of those 8 yielded the virus, the other 7 isolates were from dogs which were not clinically ill, but were housed with ill dogs. The virus was isolated from the nose of 10 animals and in 4 cases, it was isolated also from the throat.

Cell susceptibility. Virus was isolated only in cultures of dog cells (embryo and kidneys). Attempts to grow the virus in: HeLa, human amniotic, African green monkey kidney (AGMK), mouse embryo, and primary rabbit kidney (RK) cells, and continuous line of RK cells, failed. Several blind passages were carried out in AGMK, human amniotic, and rabbit kidney cells, but no virus could be recovered when tissue fluids were added to dog cell cultures.

Susceptibility to physical and chemical agents. Heat (56°C), pH 3.0 and ether treatment of fluid containing $10^4 TCID_{50}$/ml of virus, each resulted in total loss of infectivity.

Cytopathology. CPE attributable to the virus was noted in cultures of dog cells one day after inoculation. CPE occurred as multiple foci containing cells with nuclear swelling (Fig. 1). Subsequently, the foci enlarged and the affected cells were gradually detached from the glass. Examination of stained preparation revealed characteristic changes. Some of the affected cells exhibited intranuclear inclusion bodies which in most cases were not well defined but a few had halos surrounding them (Figs. 2 and 3). Basophilic granules of nuclear chromatin were arranged marginally at the nuclear membrane. Necrotic cells with pyknotic nuclei and shrunken cytoplasm were common. No giant cells or syncytia were observed either in dog embryo cells or in DK cells. The CPE was identical in cells derived from dog embryo and from DK and remained unaltered upon successive passages of the virus.

TABLE III. The Development of Neutralizing Antibodies to Herpescanis Virus Following Inoculation.

Dog no.	Prior to inoculation	Days after inoculation			
		17	30	80	130
11	<1/4	1/8	1/32	1/64	1/32
12	<1/8	1/8	1/128	1/32	1/8
16	<1/4	1/16	1/32	1/32	1/8
20	<1/4	1/16	1/64	1/32	1/8
22	<1/4	1/4	1/32	1/16	1/4
12[a]	<1/4	<1/4	<1/4	<1/4	ND
21	<1/4	<1/4	<1/4	<1/4	ND

[a] Dogs no. 12 and no. 21 were not inoculated; ND = not done.

Hemagglutination. No agglutination of dog or rhesus monkey erythrocytes was observed when incubated in the presence of virus at 37°C, 22°C, and 4°C. Agglutination of rat erythrocytes was observed at a viral dilution of 1:4 when preparations were incubated at 4°C.

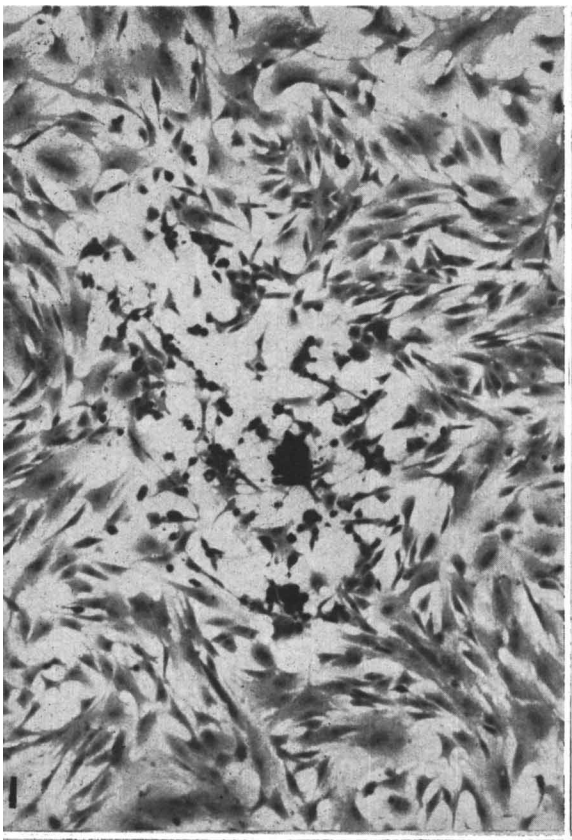
Determination of type of nucleic acid. DK monolayers infected with the isolated dog virus and herpessuis virus were exposed to media containing different specified compounds. After 1 day, all tissue cultures infected with herpessuis virus were completely destroyed, regardless of the treatment. A minimal CPE was noted after 1 day in cell cultures infected with canine virus. On the following day advanced CPE was noted in the canine virus infected cells incubated with maintenance medium or medium containing BUDR plus thymidine. Infected cells incubated with BUDR alone exhibited a slight CPE. The results of viral infectivity titers of the whole

FIG. 1. DK monolayer showing a focus of degeneration. H & E; 160 ×.

FIG. 2. DK cells demonstrating margination of granular chromatin and intranuclear inclusion bodies. Note that the inclusion bodies are not well surrounded by a definite halo. H & E; 1200 ×.

FIG. 3. High magnification of intranuclear inclusions in DK cells. H & E; 3000 ×.

FIG. 4. Broncheal epithelium of experimentally infected 2-weeks-old pup, showing intranuclear inclusion bodies. H & E; 750 ×.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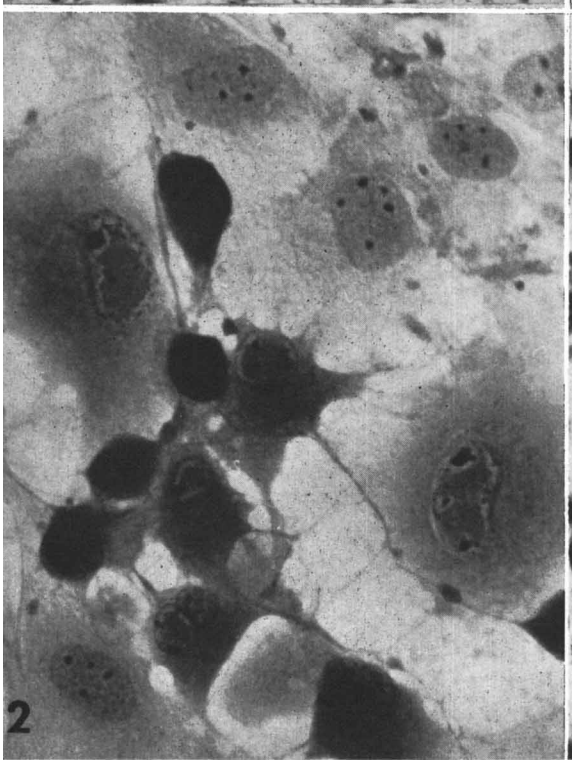
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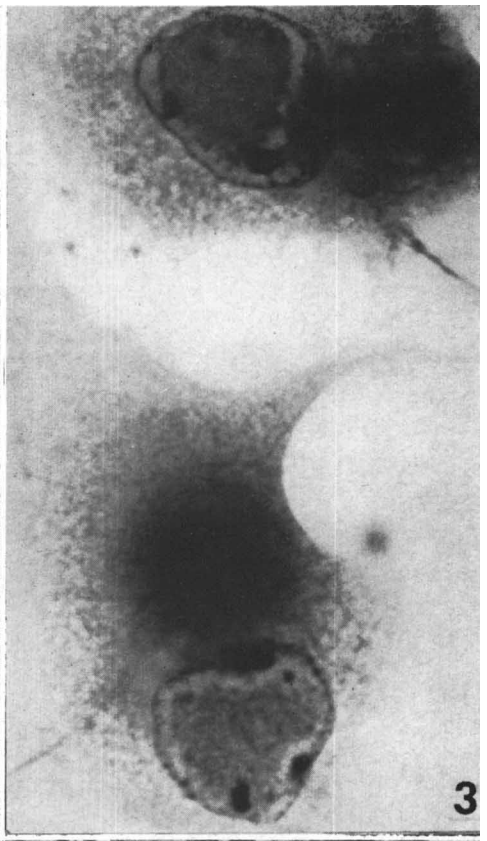
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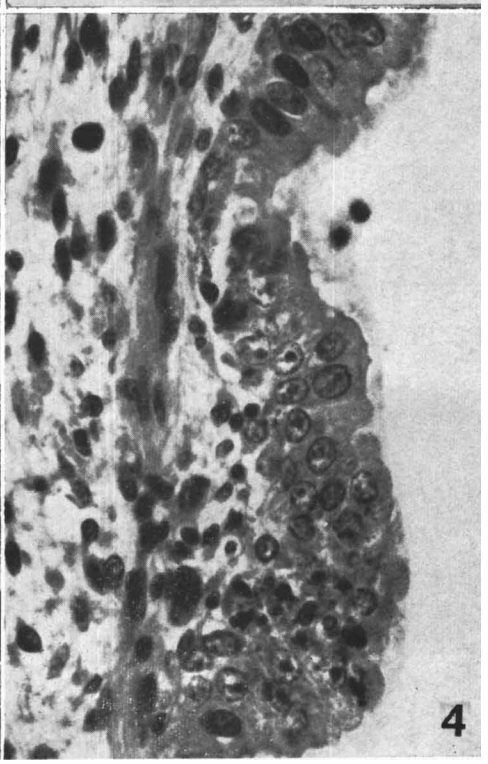
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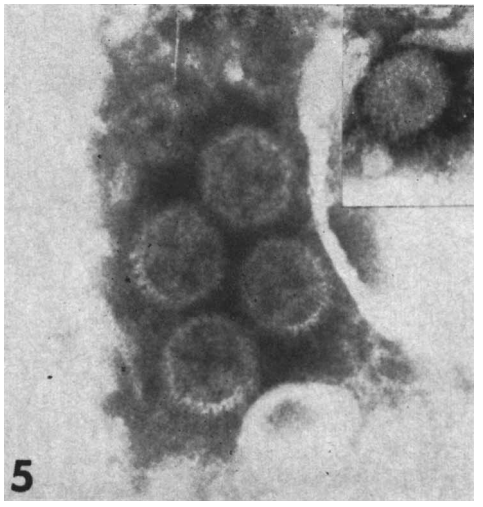

FIG. 5. Electron micrographs of negatively stained viral capsids showing hexagonal profile and projecting hollow capsomers. 120,000 × "Inset" electron micrograph of surface of negatively stained capsid showing the subunits. 98,000 ×.

cultures obtained after treatment of infected cells by BUDR are summarized in Table I. It was apparent that BUDR at doses of 20 μg/ml inhibited the infectivity and multiplication of herpessuis virus, as was expected, and also the isolated canine virus. The inhibitory effect of this dose of BUDR was suppressed by 50 mμ of thymidine/ml. Herpessuis virus and the canine virus were inhibited to approximately the same degree.

Size determination. Results of ultrafiltration studies with membrane filters showed that infective virus passed through 450 mμ and 220 mμ pores. No infectivity was detected in filtrates passing through membranes of 100 mμ and 50 mμ pore sizes.

Electron microscopy. Electron micrographs of negatively stained preparations revealed particles consisting of a body, the capsid, which appeared to be hexagonal and composed of hollow capsomers projecting outwards (Fig. 5). PTA had penetrated capsids to a varying extent. The images are not inconsistant with those published by others for herpesvirus (5). Enveloped forms could not be detected. The envelopes were probably broken away during alternate freezing and thawing followed by ultracentrifugation.

Neutralization tests. The experimental dogs, inoculated intranasally with the isolated virus, presented significant titers of neutralizing antibodies during the course of their upper respiratory disease (Table III). Sera obtained prior to viral inoculation did not possess any neutralizing antibodies while sera taken 1 month later exhibited titers that ranged between 1/32 to 1/128 in neutralization tests. The infectivity of herpescanis virus isolated by Dr. Carmichael, was neutralized to approximately the same degree by these sera. Similarly the herpescanis antiserum provided by Dr. Carmichael neutralized our virus isolated.

No antibodies were demonstrated in rabbits' sera after inoculation of herpescanis virus.

Susceptibility of laboratory animals. No lesions or manifestations of disease were observed in any of the laboratory animals held for more than 2 months after inoculation. No attempts were made to isolate the virus from these animals.

Experimental infection of dogs. The dogs that were inoculated intranasally developed respiratory signs, ranging from mild nasal discharge and sneezing to paroxysmal coughing. Table II indicates that the virus could be recovered from all inoculated dogs. All five inoculated dogs yielded the virus from the nose while three of these dogs also yielded virus from the throat, and two from anal swabs. The virus could be recovered up to the 18th day after the initial nasal inoculation and beyond this point no virus was recovered. Table III outlines the development of neutralizing antibodies, in the inoculated dogs. Previous to inoculation no antibodies were detected. Following a single intranasal inoculation a significant development of antibody titer was detected which reached a peak after a month. Following this peak the antibody titer gradually declined as indicated in Table III. The sera of the two control dogs were negative throughout this period.

The histopathological studies revealed intranuclear inclusion bodies in the respiratory epithelial cells (Fig. 4). Details of the pathology of the disease are given in another paper.

Two of the three inoculated pups died 10 days after the intraperitoneal injection of the virus and demonstrated the lesions of acute hemorrhagic disease previously described (4).

Discussion. Infectious canine tracheobronchitis is a contagious disease of world wide distribution. Previous investigators excluded the possibility of bacteria or mycoplasma as the cause of the disease (8). Clinically the disease is characterized by a variety of upper respiratory signs the paramount of which is a dry paroxysmal cough. For this reason the disease has commonly been referred to as "kennel cough". Because of its contagious nature, the disease is of prime importance in animal hospitals and other facilities where large numbers of dogs are housed together.

During the investigation of the etiology of canine tracheobronchitis, a cytopathogenic agent has been isolated from dogs which were housed with such animals, but were themselves not clinically ill. All isolations were made from nasal swabs. However, in several dogs, the virus was recovered from throat swabs and in the case of one of the dogs that were sacrificed it was also recovered from other parts of the respiratory tract, including the lungs. Histological examination of tissues from the latter dog revealed intranuclear inclusions in the trachea and bronchial epithelium. Attempts to demonstrate inclusions in the other two dogs sacrificed failed. Intranuclear inclusions were, however, demonstrated in young pups that were inoculated intranasally with the isolated virus.

The finding that BUDR inhibited the propagation of the newly isolated virus did not necessarily prove that the virus is of a DNA nature, but this observation, combined with the other properties described, is compatible with the suggestion that this virus belongs to the herpes group.

Dogs which were inoculated only once intranasally with the virus developed high titers of neutralizing antiserum which neutralized herpescanis virus isolated by Dr. Carmichael. Cross neutralization tests with other members of the herpes group were not undertaken, because the particular growth properties of the isolated virus excluded any close relation to those viruses, and since Dr. Carmichael did not find any serological relationship to his isolate with several herpes viruses tested.

From the preliminary experiments with inoculated dogs, it was found that the clinical course of the disease varies from a virus carrier state with only slight clinical symptoms, e.g., runny noses, sneezing, etc., to a paroxysmal cough which lasts for approximately 2 weeks. The virus when injected intraperitoneally into 4-day-old puppies, caused fatal hemorrhagic disease in two out of three animals injected. In these immature hosts the pathological changes were similar to those described previously by others (6 and 7). Thus, it would seem that the same virus may be associated with entirely different clinical and pathological signs.

The isolations of a virus similar to herpescanis from fetal and neonatal dogs which died of acute hemorrhagic disease (7) and of an identical virus that caused spontaneous degeneration of primary dog kidney tissue cultures (9) have been reported. The presence of herpescanis virus in fetuses suggests an intrauterine transmission of the virus. The recovery of this virus as a latent agent from kidney cells of apparently normal puppies suggests that the virus may be carried latently.

There is no statistical information concerning the incidence of fatal hemorrhagic disease in puppies but it is rarely reported. Upper respiratory infections including infectious canine tracheobronchitis on the other hand, present frequent clinical problems.

Since the causative agent tracheobronchitis has not been described before, it has not been possible to carry out controlled epidemiological studies nor has it been possible to determine the natural distribution of the virus among the dog population. It is now apparent that virus can be isolated from naturally infected dogs which do not exhibit the characteristic clinical symptoms of tracheobronchitis. That clinically inapparent infection may occur is also demonstrated by our observations in experimentally infected dogs. From our findings, which are admittedly preliminary, it is possible that the clinically characterized signs of tracheobronchitis may represent only the most severe effects of the disease in adult dogs. Some dogs may undergo an inapparent illness, after which they may become immune. However, our present results indicate that a dog which appears to be healthy may carry the virus for a time during which it may be a source of infection for susceptible dogs.

Since clinical signs of upper respiratory infections in dogs vary from a slight rhinitis to severe tracheobronchitis which at times progresses to bronchopneumonia, it is possible that the primary viral lesions in the nasal, bronchial, and tracheal mucosae predispose the affected animal to secondary bacterial infections.

Summary. The isolation of herpescanis virus from the respiratory tract of naturally infected dogs is reported. Several animals from which the virus was isolated exhibited the characteristic symptoms of canine tracheobronchitis while others were not clinically ill.

Except for differences in *in vivo* and *in vitro* cytopathogenicity, the virus possesses similar physical, chemical, and morphological characteristics of the herpes group of viruses. It was positively identified by serologic methods as herpescanis virus.

Experimental reproduction of the respiratory disease was observed following intranasal inoculation of susceptible dogs, while inoculated pups died of acute hemorrhagic disease following intraperitoneal inoculation.

The pathogenicity of herpescanis virus is evaluated on the basis of our studies and those of other workers.

We wish to express our sincere thanks to Miss E. C. Adams of the Department of Pathology, Harvard Medical School for preparation of the electron micrographs and to Dr. L. E. Carmichael of the New York State Virus Research Institute for supplying us with herpescanis antiserum.

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An Improved Method of Preparing Haptoglobin Polypeptide Chains Using Guanidine Hydrochloride* (32619)

SIAMON GORDON, HARTWIG CLEVE, AND ALEXANDER G. BEARN
Rockefeller University, New York, N.Y.

Haptoglobin is an α_2 serum protein which has the property of binding hemoglobin stoichiometrically. Genetic studies of this protein in man have revealed a striking polymorphism of three main phenotypes, viz. haptoglobins 1-1, 2-2 and 2-1 (1). All 3 genetic types consist of two kinds of polypeptide chain linked by interchain disulfide bonds, a larger β and a smaller α chain[1] (2). In the case of haptoglobin 1-1 chain fractionation has been accomplished after reductive cleavage and a tentative model has been proposed for this molecule (3). Studies of haptoglobin 2-2 and 2-1 have not yet revealed the exact arrangement of β and α chains in these phenotypes.

Chemical studies of the α chains of all 3 types of haptoglobin have been in progress for several years (4,5). There has been little detailed study of the β chains, although there is evidence that isolated β chains prepared under certain conditions are still capable of binding hemoglobin, whereas isolated α chains are inactive (6). One difficulty has been the

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[1] Abbreviations used in this paper: haptoglobin—hp; chains of each common type of hp denoted by subscript, e. g., $\beta 2\text{-}2$; $\alpha 2\text{-}2$; hemoglobin—hb.