present. Evidently these effects proceed at maximal rate in ordinary air, and are not accelerated by 100% O_2, or diminished by additional components of cigarette smoke.

Analogously, (+) ions require more than a certain minimum of CO_2 to exert their effects. However, increases in CO_2 concentration above that of ordinary air do increase the rate at which (+) ions produce their effects.†

The agent in cigarette smoke that causes reversible lowering of ciliary rate is not known. Whatever it may be, its action is effectively neutralized by (−) ions, which raise the ciliary rate just as well in a heavy atmosphere of cigarette smoke as they do in fresh air.

On the other hand, the reversible drop in ciliary rate induced by cigarette smoke alone is quickly lowered still more and made permanent by (+) ions; "permanent" in the sense that the low rate will continue indefinitely after smoke and (+) ions have been removed, although it can still be reversed by (−) ions. The extent of the (+) ion lowering in cigarette smoke is the same as in ordinary air; the rate of lowering is 3-10 times faster in cigarette smoke. Whether this more rapid rate of action of (+) ions in smoke is due to increased CO_2 concentration alone, or involves other components of cigarette smoke as well, remains to be established.

In experiments performed in humid air about 1 x 10^9 ions/cm^2/sec, as measured with the Beckett probe and Keithley electrometer, impinged on tissue surface. Ion density measurements did not vary greatly from this figure when N_2, O_2, or CO_2 were substituted for air, but this does not signify that equivalent numbers of ions necessarily were produced from the various gases, for the ambient atmospheres were far from pure. Among sources of pollution were 1) air seepage into the plastic chamber, 2) impurities in the commercially-made gases which were bled from metal tanks, 3) water vapor purposely introduced to keep the tissue moist. Nevertheless, these gases did not seem to introduce any complicating factors, presumably because the physiologically active components (O_2 and CO_2) were present in quantities below their threshold levels.

† In ordinary air, rate of (+) ion action is significantly higher *in vivo* than *in vitro*. (+) ion action mediated through additional CO_2 from expired air of living animal is evidently sufficient to cause this difference.

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Infectious Canine Hepatitis Virus Studies with Special Reference to Passage of Raccoon Tissue Cultures. (24062)

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Previous reports have described growth of infectious canine hepatitis (ICH) virus in tissue cultures derived from dog kidney(1,2,3). More recently, it has been shown that ICH virus grows in tissue cultures of ferret and pig kidney origin(4). Green *et al.* reported transmission of fox encephalitis virus infection to 1 of 14 raccoons, *Procyon lotor,* by intracerebral injection, while raccoons given virus by intraocular route were routinely infected(5), indicating that certain cells within the ocular tissues and central nervous system of these animals support growth of virus. Recently it was shown that there are no sero-

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logical differences between ICH and fox encephalitis viruses(6).

This paper deals with: 1. Cultivation of ICH virus in tissue cultures derived from wild raccoons; 2. Isolation of ICH virus from kidneys of healthy dogs; and 3. Distribution of specific ICH antibodies in sera of dogs in rural areas of Kansas.

Materials and methods. Virus strain. The infectious canine hepatitis (ICH) virus used in this study was received from Dr. James A. Baker, Veterinary Virus Research Institute, Cornell University, Ithaca, N. Y. It was received as frozen spleen. The virus was passed once in antibody free puppies by intraperitoneal (IP) injection of 1 ml of 10% ground spleen in phosphate buffered saline. When the infected puppies were moribund, the spleens were harvested and frozen at –70°C until used. *Animals.* From 1953 to 1957, 216 puppies were bled by cardiac puncture. The sera were stored at –4°C until used. The age of puppies ranged from 3 to 7 months. Puppies were received from farms in Central and Northwest Kansas. The number of puppies/litter ranged from 3 to 9. Wild adult raccoons were obtained from Iowa, Nebraska and Florida. One collared Peccarey, *Tayassu angulatus,* was obtained from Texas. Other animals were obtained from local animal dealers. Raccoons were routinely bled at the time they were sacrificed for tissue culture studies. *Roller tube tissue cultures* were prepared by 2 methods. (1) Tissue cultures were prepared from explants of the tissue of choice or (2) animal kidneys were trypsinized according to the method of Younger(7). Trypsinized, packed kidney cells were used in final dilution of 1:400. All cultures were grown in 1 ml of medium #199(8) with 2% horse serum and 3% $NaHCO_3$. Penicillin and streptomycin were present at final concentration of 100 units and 50 mg/ml respectively. Growth medium was replaced on cultures 3, 4, or 5 days after preparation as indicated by a lowered pH. Viral inoculum was added to cultures after medium change without subsequent changes in culture conditions. All infected and control cultures were observed every other day for 7 days or until cytopathologic effects (CP) occurred. Virus infected tissue culture fluids were stored at either –4°C or –70°C. Four to 8 cultures were inoculated for each virus passage. Each tube was injected with 0.1 ml of virus fluid. Virus titrations were carried out in trypsinized raccoon or dog kidney cultures. Medium #199 buffered with $NaHCO_3$ was employed as diluent for both virus and serum. Infective end points were calculated by the Reed-Muench formula(9). *Standard ICH Immune Serum.* Puppies free of ICH immune antibodies by the neutralization test were injected IP with 1 ml of 10% ICH virus infected dog spleen. Four weeks later one survivor was bled and serum stored at –4°C until used. The ICH convalescent serum had a neutralizing titer of 1:5120 against 320 $TCID_{50}$ of the virus while the preinoculation serum had a titer of less than 1:2. *Serological procedures.* Identification of either ICH virus or specific ICH antibody in serum was made by neutralization tests. The titer of standard ICH immune sera was determined by mixing 2-fold falling dilutions of serum with 100-320 $TCID_{50}$ dog kidney tissue culture (DKTC) virus. The highest serum dilution preventing CP was designated as the titer. Assay of farm puppy sera for ICH antibodies was carried out by mixing 32-320 $TCID_{50}$ DKTC virus with either 1:2 or 1:5 final dilution of serum. In the ICH antibody assay of raccoon sera 10-100 $TCID_{50}$ DKTC virus was employed against final dilution of 1:5 serum. DKTC virus below the tenth passage level was employed for assay of all sera for ICH antibody. Paired serial 10-fold dilutions of DKTC or raccoon tissue culture virus were prepared, the initial one being 1:5 dilution. Suitable dilutions of virus were mixed with equal volumes of preinoculation and convalescent sera in final dilution of 1:2 and 1:10 respectively. All serum-virus mixtures were agitated and incubated at room temperature for 1 hr. Each mixture was inoculated into at least 2 tissue culture tubes in a volume of 0.1 ml each. All sera were heated at 56°C for 30 minutes just before use. Virus titrations were always carried out simultaneously with each neutralization test. Uninoculated and serum control cultures were always included in the test. Cultures were observed daily for 7 days or until

TABLE I. Results for Neutralization of 4th Passage ICH Virus in Various Raccoon Epithelia.

Standard serum (dog)	Source of epithelium: Kidney	Corneal (endothelium)	Ventrical (ependymal)	Bladder (transitional)
Normal	3/3	3/3	3/3	3/3
Immune	0/3	0/3	0/3	0/3

Numerator = No. of tubes showing cytopathology. Denominator = No. of tubes inoculated.
4th passage raccoon kidney virus titered $10^{-4.5}$/ml; other tissues not titered.
Final dilution of normal and immune sera was 1:2 and 1:10, respectively.

CP occurred. *Results. Passage of ICH virus.* Dog kidney explant roller cultures were each inoculated with 0.1 ml of 10% emulsion of dog spleen infected with ICH virus. Widespread CP occurred in cultures on 4th and 5th days. The fluids were harvested, pooled and passed a second time. CP was apparent on 3rd day and complete on 4th day. In this manner DKTC virus was serially passed 12 times. The next 6 passages were achieved in trypsinized roller tube cultures with similar results. The 18th passage DKTC virus constituted the inoculum for initiation of the raccoon kidney tissue culture (RKTC) virus passage series. Cultures of raccoon *kidney explants,* scrapings from urinary bladder, corneal endothelium and brain ventricle ependymal cells were inoculated with 1:10 dilution DKTC virus. Widespread CP was present in all cultures in 4 days. The second to 4th passages were carried out in a similar manner with the same result. The 4th passage series of cultures were prepared in duplicate and inoculated with serum-virus mixtures of normal and standard immune dog serum. The results in Table I show that the cultures derived from different raccoon tissues all support growth of raccoon tissue culture virus. CP produced in these cultures by ICH virus was similar to that previously reported(1) for dog kidney cultures. *RKTC virus* and bladder passage virus were serially transferred to 40th and 7th passage levels respectively. Bladder virus in 7th passage with titered 10^4 $TCID_{50}$ ml in DKTC was discontinued. Results for comparative infective titers of RKTC and DKTC viruses in raccoon and dog kidney cultures are illustrated in Table II. They show that there are no significant differences in the amounts of either virus produced in either type of culture. Other tissue cultures of rabbit and hamster kidney and bladder epithelium; fetal bovine kidney, tongue and bladder epithelium, mouse mix (kidney, spleen, liver, lung), peccarey, monkey and fetal pig kidney were inoculated with RKTC virus below 18th passage level. On the 4th day after inoculation, the peccarey and fetal pig kidney cultures all showed widespread CP whereas controls remained unchanged throughout 7-day observation period. No apparent CP occurred in any of the other cultures even when they were incubated for additional 7 days. Second passages were carried out with the same results. Peccarey and fetal porcine kidney virus passages were discontinued. *ICH Isolates.* During this research, a characteristic CP appeared spontaneously in uninoculated trypsinized dog kidney cultures prepared from 2 different dogs. In the first instance, the puppy had no known history of exposure or disease. The second dog had been exposed to ICH disease 3 months before its kidneys were removed and trypsinized. The incidence of spontaneous CP in cultures was one in every 15 tubes examined. Standard immune dog serum and serum of the first dog in final dilu-

TABLE II. Results for Titration of Tissue Culture ICH Virus of Raccoon and Dog Kidney Origin in Raccoon and Dog Kidney Cultures.*

RKTC virus			DKTC virus		
	Substrate			Substrate	
Pass.	RK	DK	Pass.	RK	DK
8	$10^{-4.5}$	$10^{-4.5}$	3		10^{-4}
9	"	10^{-4}	9	$10^{-4.5}$	10^{-5}
14	NT	"	17	$10^{-4.0}$	10^{-5}
19	10^{-4}	NT	18	$10^{-4.5}$	$10^{-4.5}$
25	$10^{-4.5}$	"			
34	10^{-5}	"			
40	10^{-4}	10^{-4}			

* RKTC, DKTC = Raccoon, dog kidney tissue culture virus. RK, DK = Raccoon, dog kidney cultures.
NT = Not tested.

TABLE III. Results for Presence of Neutralizing Antibodies against ICH Virus in the Sera of Puppies and Raccoons.

Serum source	Age (mo)	No. sera	Serum antibodies* Positive	Negative
Raccoons	Adult	23	1	22
Puppy	3–7	216	17	199

* Dog sera tested in a final 1:5 dilution or less against 32-320 $TCID_{50}$ virus. Raccoon sera tested in a final 1:5 dilution against 10-100 $TCID_{50}$ virus. DKTC virus used in all tests.

tion of 1:10 neutralized at least 1000 $TCID_{50}$ of homologous ICH isolate and DKTC virus. Serum of second dog was not tested for virus neutralization.

Results for serum survey for ICH antibodies. Neutralizing substances were found in serum of 1 of 23 raccoons against 10 to 100 $TCID_{50}$ DKTC virus. The results shown in Table III indicate under the limited number of sera tested, that less than 5% of raccoons show evidence of ICH infections. Less than 9% of sera from farm puppies tested showed presence of specific antibodies against ICH virus.

Final identification of the RKTC virus at 39th passage level was by direct neutralization with standard immune serum and the inoculation of antibody-free puppies with virus.

Results for the serum-neutralization test showed that standard normal dog serum in final 1:2 dilution failed to neutralize RKTC virus while specific immune serum in final 1/10 dilution neutralized at least 4 logs of the virus.

Nine antibody-free puppies were divided into 2 groups. Each of 5 puppies in the first group were injected intramuscularly with 0.5 ml of RKTC virus. The second group served as contact controls and were housed with puppies in Group 1. In the first group temperature elevation of 102.2°F or above commenced 2-4 days after inoculation, persisted 9-15 days and then dropped below 102.2°F. Three puppies gained weight throughout 28-day observation period. The other 2 puppies developed unilateral corneal opacities, lost weight and 1 died on 19th day of infection. Convalescent serum was not obtained from this dog. Lesions pathognomonic for ICH disease were not found at autopsy. Severe bacterial pneumonia was found in both lungs and hemolytic streptococci and coli were recovered from both liver and spleen on blood agar plates. It was concluded that this dog did not die of ICH disease.

In the second group temperature elevations above 102.2°F commenced after 12 days of contact with Group I, persisted for 3-11 days, and returned to 102.2°F or lower. All puppies gained weight and remained essentially normal throughout 28 days of observation. Temperature elevations in either group of puppies did not exceed 104°F. Persistence of temperature elevations in the 4 puppies in Group II was 4-6 days less than the inoculated puppies in Group I indicating that ICH infection in these dogs was more mild than in Group I.

Results of serum-neutralization studies for 9 preinoculation and 8 convalescent dog sera against DKTC virus showed that specific ICH antibodies appeared in convalescent sera of both groups. The 9 pre-inoculation sera were negative for ICH antibodies in final dilution of 1:2 against 320 $TCID_{50}$ DKTC virus while the convalescent sera in final dilution of 1:10 neutralized at least 3 logs of virus. Appearance of circulating antibodies in blood of puppies in Group II established the transmission of virus by direct contact from puppies in Group I with subsequent development of inapparent ICH infections.

In other studies, it was shown that dogs recovered from infection with RKTC virus and their contact controls were solidly immune against virulent challenge spleen virus while 30% of the non-immune controls died.

Summary. 1. Infectious canine hepatitis (ICH) virus grown and passed 39 times in tissue cultures derived from wild raccoons. The tissue culture virus in 39th passage was neutralized by specific immune serum prepared against the original dog spleen virus. When the 39th passage virus was introduced into antibody free puppies, all developed a mild ICH infection resulting in appearance of specific serum neutralizing antibodies in blood against dog kidney tissue culture virus. 2. Eight percent of the puppies, 3-7 months of age, obtained from farms in Central and Northwest Kansas, have specific neutralizing

antibodies against ICH virus; ICH antibodies in a 1:5 titre were found in 1 of 23 sera from wild raccoons. 3. ICH virus was isolated from dog kidney tissue cultures derived from 2 apparently healthy dogs. In one instance, specific ICH neutralizing antibody was present in the blood.

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Antagonistic Action of Liver Amino Acids and 2,4-Dinitrophenol on Growth of *Escherichia coli*. (24063)

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It is known that extracts and fractions of liver protect animals from toxic effects of poisons and hormones. Ability of liver extracts to counteract the toxic activity of thyroid powder and 2,4-dinitrophenol (DNP) on various living systems is an example of this type of antagonism and is of particular interest because DNP and thyroxin are both inhibitors of oxidative phosphorylation. Crude liver preparations and components of liver extracts reverse thyroid stress occurring in rats fed with purified diets containing thyroid powder or iodized protein(1). Attempts have been made to identify the unknown factors (2,3) responsible for this anti-thyrotoxic activity, or to refer such activity to known components of the liver(4,5). An antagonism of liver extracts against inhibitory action of DNP has been described in studies with mammalian tissue homogenates(6), as well as with bacteria(7). Analysis of growth-stimulating and anti-DNP activity of liver extracts in bacterial systems shows that several fractions are involved: one is composed of bile salts; another of liposoluble vitamins; a third of steroids; a fourth of unsaturated fatty acids and possibly hydroxy-acids; and a fifth of amino acids and oligopeptides. The hypothesis has been expressed that these substances represent physiological antagonists of thyroid hormones in mammalian tissues, at a cellular level. Similar results were obtained by other authors(4,5) using metabolic-rate test of rats fed with iodized protein for titrating the anti-thyrotoxic activity of liver components. The present work is concerned with the amino acid fraction of liver extracts.

Technics. Liver-amino acid preparation was obtained as follows: Cow's liver, homogenized in Waring Blendor and autolyzed under toluene at 37°C, was dried, pulverized, and extracted by refluxing twice each with boiling ethanol followed by hot water. The residue of coagulated proteins was washed several times with H_2O and hydrolyzed with 6*N* HCl at 100°C for 18 hours. HCl was removed by vacuum distillation and the residue brought several times to dryness after addition of water. The dry material was then dissolved in H_2O. The resulting preparation had a nitrogen content of 13 mg/ml and was used without neutralization for chromatographic analysis, or was neutralized with KOH and sterilized for microbial assays. Medium A used for *E. coli* strain B had the following

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