Biophysical Characterization of Infectious Bovine Rhinotracheitis Virus. (24437)

A. J. TOUSIMIS, W. V. HOWELLS, T. P. GRIFFIN, R. P. PORTER,*
W. J. CHEATHAM† AND F. D. MAURER
Armed Forces Inst. of Pathology, Washington, D. C.

The agent of infectious bovine rhinotracheitis (IBR), an acute upper respiratory disease of cattle, has been isolated from nasal washings of infected cattle on bovine kidney tissue culture. The viral nature of the etiologic agent has recently been established by extensive serologic and transmission studies(1-5). The virus is cytopathogenic to cells of both bovine kidney and human amnion cultures in which it induces development of intranuclear inclusions, readily observed in stained preparations(6). The agent is relatively stable and can be held at 4°C over 30 days and at 22° C for 3 days with no measurable loss of titer(7). This study was undertaken to demonstrate the entity associated with the infectious unit and to determine its physical characteristics.

Materials and methods. Tissue culture. Bovine kidney cell cultures were prepared by the method of Madin *et al.*(8) with a medium of 0.5% lactalbumin hydrolysate in Hanks balanced salt solution containing .01 M Tris buffer, with addition of 5% lamb serum. Human amnion cell cultures were prepared by the method of Zitcer *et al.*(9) with a medium of bovine amniotic fluid containing 5% horse serum. *Virus.* Pritchard and American strains of IBR virus as described by Cheatham and Crandell(6) were used. Virus pools of Pritchard strain were prepared by inoculating the agent in bovine kidney cultures into 32 oz. prescription bottles. When the cytopathogenic effect was complete at about 72 hours, the fluid was frozen and thawed several times to disrupt the infected cells, then centrifuged in refrigerated centrifuge at 2,000 rpm for 10-15 minutes to remove cellular debris. The supernate was centrifuged at 20,000 rpm in No. 21 rotor of Spinco model "L" preparative centrifuge for one hour; the sediment was resuspended in phosphate buffered saline of pH 7.4 and again centrifuged at 5,000 rpm for 15 minutes in the same rotor. The infectious fluid was dispensed into glass ampules, sealed and stored at −60°C. Ten-fold serial dilutions of the virus pool, 10^{-1} through 10^{-7}, were made in tissue culture medium. One-tenth ml volumes of each dilution were inoculated into each of 4 bovine kidney tissue culture tubes. The tubes were observed daily 5 days until a cytopathogenic end point was reached. The virus pool titered $10^{-6.5}$/0.1 ml throughout experimental period. Tissue culture infective dose 50% ($TCID_{50}$) was calculated by the method of Reed and Muench(11). Both analytical and preparative Spinco centrifuges were used for identification of infectious particle. Since concentration of the infectious agent was not high enough to permit optical detection of the sedimenting boundary in the analytical centrifuge cell, pseudovelocity procedures were employed using methods described previously(13) for identification of the virus particle. Experiments in the preparative Model "L" centrifuge were carried out using the SW-39 rotor. Centrifugation speeds were 5,000, 10,000, 15,000, 20,000, and 25,000 rpm for each of 10, 15, 20 and 25 minute periods. At the end of each run, samples were withdrawn from each third of tube (top, middle and bottom), and titrated. Using standard centrifugation relations(12), sedimentation coefficients‡ were computed for each level of tube at indicated velocities. A 2-log unit loss in infectivity was interpreted

* Present address: Univ. of Miami, Coral Gables, Fla.

† Present address: Dept. of Pathology, Vanderbilt Univ., Nashville, Tenn.

‡ The sedimentation coefficient $S = \left(\frac{1}{\omega^2}\right) \cdot \left(\frac{1}{t_2 - t_1}\right) \cdot \left(\frac{x_2 - x_1}{x}\right)$, where ω is the centrifugal field, $(t_2 - t_1)$ is time necessary for 2-log unit drop of infectivity in radial distance $(x_2 - x_1)$ of rotor with radius x.

TABLE I. Particle Size of IBR Virus as Determined by Ultracentrifugation.

Exp. No.	Method	Calculated virus particle diameter mμ		mμ
2 E	Pseudo-	>125		
3 E	velocity	>146	&	<166
6 E		>105 to 137		≧152 & <160
1 L	SW-39 rotor	<210		
2 L		> 95	&	≧148
3 L		>121	&	≧151
4 L		≧150	&	<210

as time necessary for virus particle to travel the distance ($x_2 - x_1$) used in these calculations. Spherical particle diameters were computed from corrected sedimentation coefficient to water and 20°C ($S_{20,w}$) of the cytopathogenic agent, the viscosity of medium (η) and the difference between particle and medium densities.§ Specimens for electron microscopy studies were prepared from stock virus by various methods, such as air drying, agar pseudoreplica(17) or fixed in formaldehyde and dialyzed against distilled water before air drying. They were shadowed with uranium at an angle of arc tan ⅓ and examined with a RCA electron microscope EMU-3B. At intervals during cytopathogenic period, human amnion cultures infected with American strain of IBR were fixed 2 hours with 1% osmium tetroxide in isotonic veronal buffer of pH 7.35 and scraped from wall of tubes. The infected cells were dehydrated in graded ethanol and embedded in methacrylate by the method of Tousimis and Hilleman(10). Ultrathin sections made with a Porter-Blum type ultramicrotome(14) were placed on grids coated with Parlodion or Formvar film and examined in electron microscope without removing the methacrylate.

Results. Centrifugation. The results indicated that the particle associated with infectivity has a spherical diameter smaller than 175-210 mμ but equal to or larger than 148-151 mμ. Calculations based on pseudo-velocity experiments carried out with partition cell(15) in the analytical ultracentrifuge, showed that the infectious particle has a hydrated diameter in the same range as that found with the SW-39 rotor (Table I).

§ Particle diameter (cm) $d = \sqrt{\frac{18\eta S_{20,w}}{\rho_2 - \rho_1}}$, where ($\eta$) is viscosity of medium, $S_{20,w}$ is sedimentation coefficient and ($\rho_2 - \rho_1$) density difference between particle and medium.

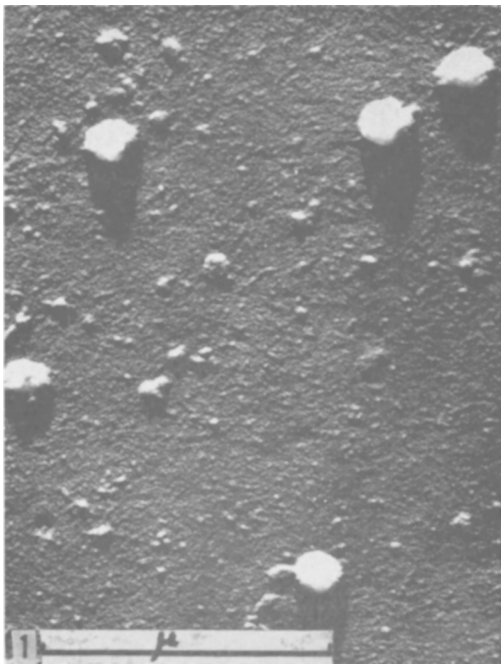

FIG. 1. Electron micrograph of IBR virus prepared by agar pseudoreplica method directly from stock solutions used in ultracentrifugation experiments without further concentration or fixation. Shadowed with uranium at arc tan 1/3. × 38,400.

Examination with the electron microscope of suspensions of the IBR agent in buffered isotonic saline, clarified from infected bovine kidney tissue culture cells, revealed particles as seen in Fig. 1. The majority of particles in these suspensions were 153 ± 12 mμ in width when prepared by agar pseudoreplica method and shadowed with uranium. On the average about 20% flattening of these particles was measured from shadowlengths. Polysterene latex spheres of 260 mμ in diameter were used to determine local shadow angles(16). Assuming that the virus has flattened to an oblate spheroid upon drying(10), its calculated diameter is 136 mμ with 10.8 mμ % standard deviation. Particles of this diameter were not found in normal control preparations.

In virus suspensions fixed in 2% formalde-

hyde before preparation for electron microscopy, particles with very little flattening and widths of 137 ± 9 mμ were observed. Many of these particles had a less electron-dense central core, 35 to 46 mμ in width, and others were irregular in shape and contained round dense masses of about 50 mμ (Fig. 2A and 2B).

In addition to the 136 mμ diameter particle. we found smaller particles of about 110 mμ in width and others of 40 mμ or less. The smaller particles (40 mμ or less) were eliminated by differential centrifugation without any detectable change in cytopathogenicity of the preparation. Particles of the 110 mμ size were also found in normal control cell homogenates. This observation is not sufficient to eliminate any possible association of these particles with the virus itself. The possibility that these are the incomplete or dissociated virus can not be excluded. The presence of high numbers of particles smaller (40 mμ or less) than the virus in infected cultures suggests that these are cellular components or the product of cell degeneration. Correlation of the number of virus particles as measured in electron micrographs with cytopathogenic units(13). showed that approximately 2 x 10^9 particles/ml correspond to infectivities of 10^7 cytopathogenic units/ml.

Electron microscopy of ultrathin sections of human amnion tissue culture cells infected with IBR virus showed numerous intranuclear particles of 125 to 140 mμ in width (Fig. 3). At later stages of infection (12 days) these virus-like particles were found in the nucleus and cytoplasm and also attached to the surface of cells (Fig. 4).

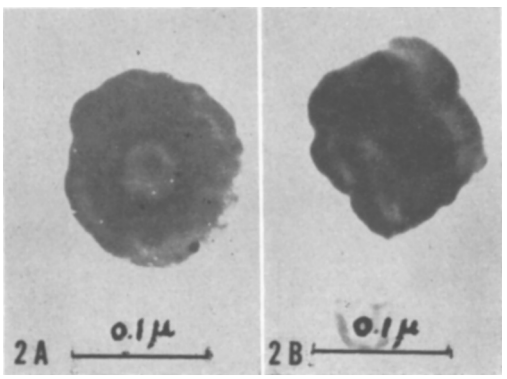

FIG. 2A and 2B. Formaldehyde-fixed virus particles for 20 min., dialyzed against distilled water for 24 hr and air dried on parlodion-coated grids. Shadowed lightly with uranium. × 180,000.

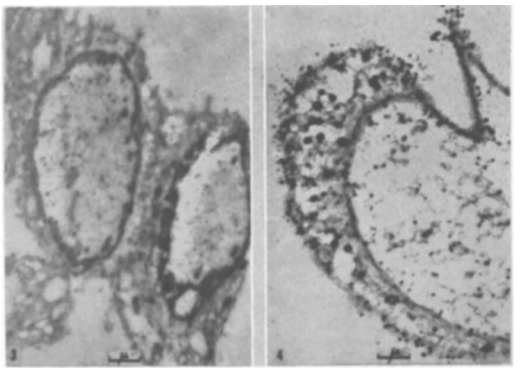

FIG. 3. Human amnion tissue culture cells 10 days after infection with IBR. Virus-like particles are seen within the nucleus. O_sO_4 stain and methacrylate embedded. × 4,600.

FIG. 4. Portion of a human amnion cell 12 days after infection. Virus-like particles appear in the enlarged nucleus and on cell surface. × 4,600.

Summary. The size of infectious bovine rhinotracheitis virus grown in bovine kidney tissue culture, based on ultracentrifugation results is between 145-156 mμ in diameter. Electron microscopical examination of infectious fluids revealed particles of 136 ± 10.8 mμ in diameter. Similar particles were observed intracellularly in ultrathin sections of the infected human amnion cells in tissue cultures.

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Comparison of Monoamine Oxidase Inhibitory Effects of Iproniazid and Its Phenyl Congener.* (24438)

AKIRA HORITA AND RALPH G. PARKER (Introduced by J. M. Dille)
Dept. of Pharmacology, School of Medicine, University of Washington, Seattle

Iproniazid (1-isonicotinyl-2-isopropylhydrazine) has gained widespread interest in studies of the metabolism of biological amines. Its ability to inhibit monoamine oxidase (MAO) has greatly facilitated the understanding of the properties of this enzyme as well as of pharmacological and biochemical effects of serotonin (5-hydroxytryptamine) and norepinephrine in the brain. The MAO inhibitory properties of iproniazid *in vivo* were earlier described by Zeller *et al.*(1) and Schayer(2). Iproniazid has also been found to be effective in treatment of depressed mental patients. Its use in these cases presumably depends upon inhibition of MAO and prevention of the degradation of endogenous amines of the brain. These and other findings have stimulated the search for newer and more potent inhibitors of MAO. Recently the compound beta-phenylisopropylhydrazine† (PIH) was found to be some 30-50 times as potent as iproniazid in inhibiting MAO both *in vitro* and *in vivo*(3). The present report describes the effect of a closely related compound, 1-isonicotinyl-2-phenylisopropylhydrazine dihydrochloride‡ (isonicotinyl-PIH) on MAO activity. Isonicotinyl-PIH also resembles iproniazid, differing only by the presence of a phenyl group on the isopropyl chain. The similarities in structure of the 3 compounds are seen in Fig. 1.

Methods. Thirty-six male Wistar rats were used. *In vitro* MAO activity was determined by the method described by Sjoerdsma *et al.* (4) and by Bogdanski *et al.*(5). One milliliter of liver homogenate, 200 mg/ml was incubated with 4 μM of serotonin creatinine sulfate at pH 7.0. The inhibitors were added to the enzyme preparations and incubated 15 minutes prior to addition of substrate. Total volume of the incubation mixture was 3 ml. All incubations were made at 38°C in a constant temperature shaker. After incubation for 30 minutes the mixtures were extracted and assayed for residual serotonin according to the nitrosonaphthol method of Udenfriend, *et al.*(6). Readings were made on a Beckman DU spectrophotometer. *In vitro* studies were carried out using concentrations up to 10^{-4} M of the inhibitors. The effect of iproniazid and isonicotinyl-PIH on liver and brain MAO *in vivo* was also investigated. A dose of 5×10^{-5} M/kg of inhibitor was injected subcutaneously 2 hours prior to sacrifice of the animals. Tissues were quickly removed and homogenized and diluted to a concentration of 20% (liver) and 33% (brain). One

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† Beta-phenylisopropylhydrazine is designated as Compound JB-516, Lakeside Labs.

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