duced vagal ganglionic inhibition and blockade of the stimulant effect of acetylcholine on the isolated rabbit heart.

Summary. Hexamethonium reduced sensitivity of adrenal medulla to nicotine, but not to potassium. A newly synthesized compound. N-N-diisopropyl-N′-n-butyl-N′-diethylaminoethyl urea (P-275) was similar to hexamethonium in its effects on the adrenal, as well as in its ability to inhibit the cardiovascular depressant effects of vagal stimulation and the acetylcholine stimulation of the isolated atropinized rabbit heart.

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Received October 1, 1958. P.S.E.B.M., 1959, v100.

Isolation of Mucosal Disease Virus by Tissue Cultures in Mixture 199, Morgan, Morton and Parker.* (24532)

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Isolation of viruses by tissue culture methods from cattle with bovine mucosal disease. infectious bovine rhinotracheitis, and virus diarrhea of New York type of mucosal disease complex(1) have been reported by at least 4 teams of investigators. Underdahl, *et al.*,(2) isolated a cytopathogenic virus from cattle having bovine mucosal disease (BMD). York. Schwartz and Estela(3) and Madin and his associates(4) isolated viral agents from cattle having infectious bovine rhinotracheitis; Lee and Gillespie(5) isolated a strain of virus from cattle with virus diarrhea New York. All foregoing virus isolations employed trypsinized cells and medium containing serum from cattle, horse or lamb. Lee and Gillespie also used roller tube explant method isolation procedures. Underdahl (personal communication) believed that his success in isolation and cultivation of a viral agent from animals having BMD depended upon using a homologous system of bovine kidney cells and bovine serum. He obtained antibody free serum by drawing blood from a newborn calf which had not been allowed to nurse. However, Schipper and Eveleth(6) report calves being born and dying 18 to 96 hours after birth showing pathology typical to BMD upon necropsy. They also report similar pathology in calves born dead at full term. In one instance, necropsy of a mature cow diagnosed as having mucosal disease possessed an 8½ month old fetus with typical lesions of mucosal disease. No BMD virus or hyperimmune serum was demonstrated from such calves since virus isolations procedures were not yet established. Since that time however, we have had a still born full term calf which showed pathology typical to BMD. While no viruses were recovered from biopsies of this calf, the serum, when diluted 1:64, was capable of neutralizing 1000

* Funds were in part provided by NC regional project 34 and Fort Dodge Labs. This is a progress report on Mucosal Disease of Cattle. The authors wish to acknowledge assistance and advice of Dr. D. F. Eveleth, Dept. of Vet. Science.

Published with approval of director, No. Dak. Agric. Exp. Station, Fargo.

$TCID_{50}$ of the BMD virus. While serum has value in promoting cellular growth *in vitro* its necessity for virus propagation is questionable. Mixture 199†(7) allowed proliferation of trypsinized bovine kidney cell cultures, thus supplying cellular metabolites that might possibly be utilized by viruses in their propagation, and eliminated the variable of serum.

Methods and materials. Bovine kidney cells were obtained by trypsinization, according to Youngner(8) as modified by Madin(9). The cortex of kidneys of bovine feti approximately 3 to 4 months of age were used as tissue source. Suspension of cells was washed 3 times and diluted 1:10 with Mixture 199 following final stage of trypsinization. 0.1 ml was then seeded directly into 125 x 15 mm screw cap test tubes containing 1 ml of Mixture 199 to which 100 units of penicillin and 100 mg of streptomycin/ml had been added. The resulting cell suspension yielded 500,000 to 800,000 cells/tube. The tubes were incubated at 36°C in stationary rack at 5° angle for 72 hours to allow cells to adhere to glass, during which time a substantial monolayer was formed. At the end of 72 hours the medium was replaced with 1 ml of fresh Mixture 199 and 0.1 ml of inoculum was added to half of the tubes. The BMD inoculum was prepared from 3 tissue sources of animals displaying symptoms prior to death and pathology typical of mucosal disease upon subsequent necropsy. One inoculum was derived from mesenteric lymph nodes, one from spleen tissue and the third inoculum was pooled tissues containing lymph nodes, spleen and whole blood. All the inoculum was processed by diluting 1:10 with Hank's solution and homogenized in a Virtis tissue homogenizer. Following centrifuging at 3000 rpm for 20 minutes the supernatant was diluted 1:100 with Hank's solution and treated with 1000 units of penicillin and 1000 μg of streptomycin/ml for one hour under refrigeration. This material was then used as tissue culture inoculum or stored at –40°C.

Results. 1. *Isolation and cytopathogenic effects (CPE) of virus.* CPE was noted in tubes containing both spleen and pooled tissue inoculum but not in tubes containing lymph node inoculum. The CPE on trypsinized bovine kidney cells consisted primarily of vacuolation. Small vacuoles appeared in the cytoplasm approximately 4 to 6 days following inoculation. Vacuoles increased in size and terminated with destruction of the cells which resulted in open patches in tissue cultures until eventually the tubes were virtually devoid of cells. Subpassages of fluid from the tubes showing CPE regularly reproduced these characteristics in tubes containing trypsinized bovine kidney cells and the CPE required approximately the same amount of time to make its appearance in such subpassages. No CPE was noted in repeated passages to tubes containing the following cell lines: HeLa cells, trypsinized embryonic bovine testicular cells, embryonic bovine skin-muscle roller tube explants, or trypsinized embryonic ovine kidney cells. Negative results were also noted from repeated passage in the following embryonic animal kidney and skin-muscle roller tube explants: rabbit, mouse, hamster and chick fibroblast. Control tubes without added inoculum were maintained in Mixture 199 for over 15 days without changing Mixture 199 or other cell feeding, and displayed no apparent cellular deterioration. To determine whether bovine tissue inoculum would in itself have CPE on trypsinized bovine kidney cells, inoculum control tubes were established. Three inocula were prepared as previously described from a calf with acute Salmonellosis upon necropsy. This calf was negative for BMD as based on the serum neutralization test. In such control tubes the inoculum had no CPE on the bovine kidney cells in either initial tubes or subsequent subpassages.

2. *Pathogenicity of tissue culture virus for calves.* Two 8-month-old Holstein calves, Nos. 976 and 979, found negative for neutralizing antibodies for BMD as based on serum neutralization test, were injected intravenously with 2 ml of tissue culture virus from the 3rd passage. Both animals were then housed in a heated, well ventilated barn for observation. A blood specimen was taken

† Mixture 199 was supplied by Microbiological Associates, Washington, D.C.

TABLE I. Neutralization of Tissue Culture BMD Virus by Antisera from Various Sources.

Serum source	Inoculum	Homologous serum dilution neutralizing 1000 $TCID_{50}$* Pre-inoculation	Convalescent
	Tissue culture virus		
Calf 976	7–799A	0	1:128
979	"	0	"
Rabbit A-31	"	0	1:64
32	8–373A	0	"
33	8–261A	0	1:32
34	8–379A	0	1:64

* All heterologous cross neutralizations were demonstrated at the same dilutions as homologous neutralization titers.

from each animal every fifth day for 2 months. From 6 to 10 days following virus injection the calves had mild temperature rises and one, No. 976, suffered a 2-day period of mild diarrhea. From 15 to 25 days post-inoculation the calves blood demonstrated neutralization antibodies for BMD virus. Absence of the more serious manifestation of bovine mucosal disease as observed in feed lot conditions, may be explained by sheltering of experimental animals from natural environmental stress factor.

3. *Serum neutralization studies.* Serum from above animals was mixed with equal volumes of tissue culture virus fluids in final concentrations of 1000 $TCID_{50}$ virus. Serum-virus mixtures were incubated at 20°C for 2 hours and 0.8 ml was inoculated into each of 10 tubes. Tests were determined when cells in the virus control tubes showed complete CPE.

Convalescent serum from calves that had been injected with virus from the 3rd tissue culture passage, diluted 1:128, neutralized 1000 $TCID_{50}$ of virus, while undiluted pre-inoculation serum from the same calves failed to neutralize the virus.

The relationship of viruses isolated from cattle having symptoms and pathology of BMD in 4 widely separated areas of North Dakota was demonstrated with serological studies conducted with rabbits. Two rabbits were injected twice intravenously with 1 ml of 8th passage tissue culture virus from each of the 4 original BMD virus isolates. Rabbits were bled 10 days before the first virus injection and 20 days following last injection. None of the pre-inoculation serum demonstrated neutralizing antibodies. However, convalescent serum when diluted 1:64 in 3 cases and 1:32 in one instance was capable of neutralizing 1000 $TCID_{50}$ of virus. In cross neutralization studies, hyperimmune serum from any of the test rabbits was capable of neutralization of all of the 4 originally isolated BMD viruses. The results of the serum neutralization studies are presented in Table I.

Summary. A virus has been isolated from cattle which displays typical symptoms and pathology of bovine mucosal disease. Virus isolations and propagation were carried on using tissue cultures of trypsinized bovine kidney cells in Mixture 199 exclusively. Two calves which were negative for neutralizing antibodies for bovine mucosal disease were injected with tissue culture virus and developed hyperimmune serum from 15 to 25 days post-inoculation. Serology conducted with laboratory rabbits indicate similarity of viruses isolated from 4 separate herds of cattle in North Dakota.

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