relatively anergic to PPD and O.T. used for skin tests. 2. Bilateral adrenalectomy does not reverse mouse tuberculin anergy.

1. Gerstl, B., and Thomas, R. M., *Yale J. Biol. and Med.*, 1940-41, v13, 679.

2. Pierce, C., Dubos, R. J., and Middlebrook, G., *J. Exp. Med.*, 1947, v86, 159.

3. Long, J. B., and Favour, C. B., *Bull. Johns Hopkins Hosp.*, 1950, v87, 186.

4. Osgood, C. K., and Favour, C. B., *J. Exp. Med.*, 1951, v94, 415.

5. Wells, A. Q., and Wylie, J. A. H., *Lancet*, 1949, v1, 439.

6. Christian, J. J., *Science*, 1953, v117, 78.

7. Dubos, R. J., and Middlebrook, G., *Am. Rev. Tub.*, 1947, v56, 334.

8. Seibert, F. B., and Glenn, J. T., *ibid.*, 1941, v44, 9.

9. Huddleson, I. F., Hardy, A. V., Devono, J. E., and Giltner, W., *Brucellosis in Man and Animals*, New York, The Commonwealth Fund, 1948.

10. Favour, C. B., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 269.

11. Fremont-Smith, P., and Favour, C. B., *ibid.*, 1948, v67, 502.

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## Propagation of Infectious Canine Hepatitis Virus in Tissue Culture. (20843)

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The successful propagation of poliomyelitis virus in tissue culture, recently reviewed by Weller(1), has led several investigators to apply the roller tube method to the study of other viruses. Thus, members of the Coxsackie group were also found capable of multiplication in monkey tissue cultures by Riordan and others(2), who observed a cytopathogenic effect similar to that obtained with poliomyelitis virus. Similarly, using a stable strain of human epithelial cell, Syverton and Scherer(3) reported the serial growth of poliomyelitis virus with cytopathogenic effects, as well as that of a variety of other viral agents.

The purpose of this communication is to describe the successful use of the roller tube method for the propagation of infectious canine hepatitis virus in dog kidney tissue cultures.

*Materials and methods. Virus.* The virus employed in this study had been used for the experimental infection of puppies, as reported in previous publications(4,5), and its origin described(5). *Tissue culture method.* Roller tube cultures of monkey, rabbit and dog kidney and of whole chick embryo in 1 ml of synthetic medium No. 199(6) containing trypsin inhibitor, were incubated at 37°C at 8 to 10 rph. The kidney tissues were grown for from 4 to 5 days and the chick embryo tissue for 2 to 3 days. The medium was renewed before infection, and no further changes were made thereafter. The inoculum consisted of 0.1 ml of 10% dog liver suspension, or infected tissue culture fluid. Infected and control tubes were observed daily for not more than 7 days. When cytopathogenic effects occurred, the infected tissue fragments and fluid were removed by scraping and suction to a Tenbroek grinder. The culture suspension thus obtained was available for either subculture or for storage in the dry ice chest. *Qualitative neutralization test.* This was done by mixing equal volumes of undiluted harvest fluids and undiluted fox immune serum previously inactivated at 56°C for 30 minutes. Controls were similarly made by mixing equal volumes of harvest fluid with either inactivated normal dog serum or inactivated ICH fox immune serum. After further incubation for one hour at 37°C, each mixture was injected in 0.1 ml amounts into groups of 3 or 4 dog kidney culture tubes. These were observed daily until complete cytopathogenic effect had taken place in the normal dog serum control tubes.

*ICH fox immune serum.* The standard serum was prepared in foxes as follows: seven

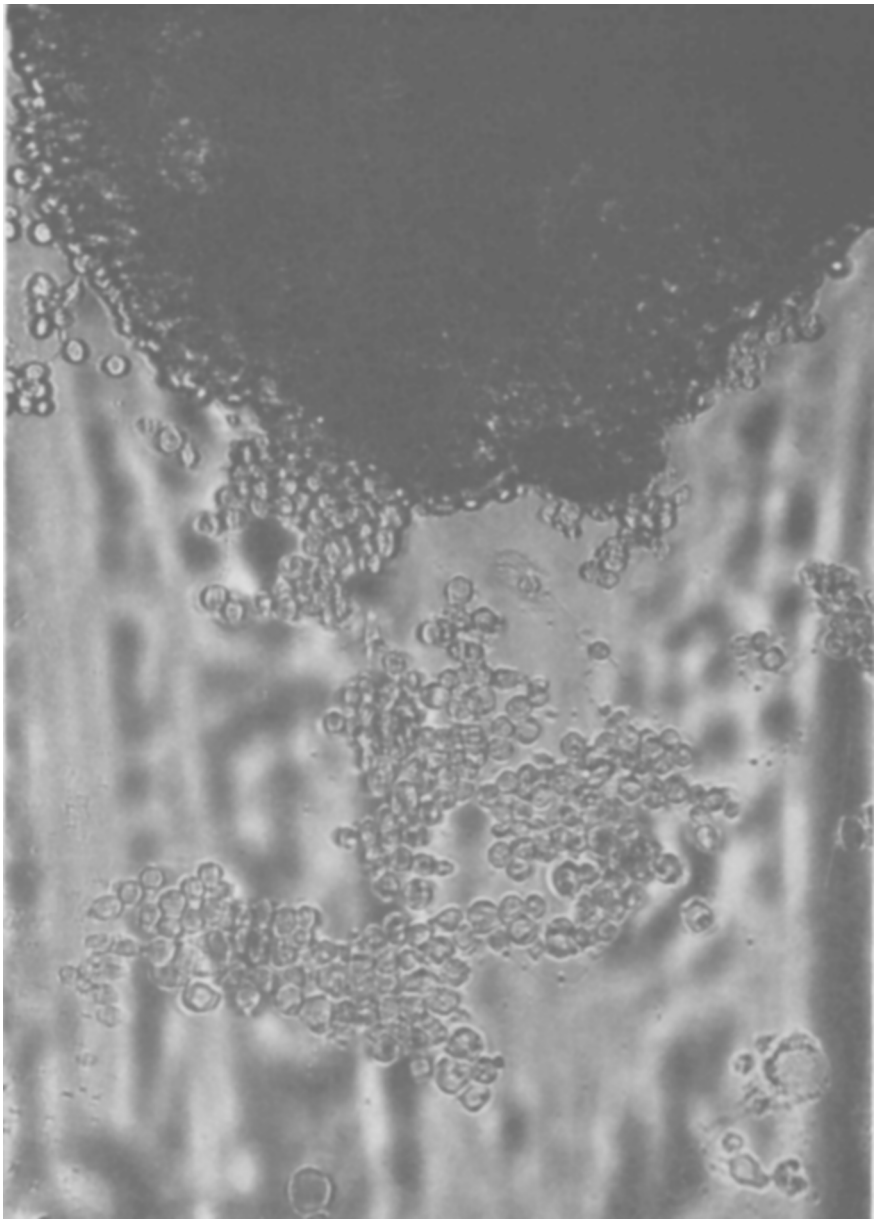

FIG. 1. Dog kidney tissue culture showing cytopathogenic effect following infection with ICH virus. × 175.

ranch-bred foxes were given repeated injections of a 20% dog liver suspension over a period of 3 months. They were bled out 14 days after the last injection. Their blood was pooled, the serum separated and stored in 25 ml amounts in rubber stoppered glass vials in the electric freezer. Repeated titrations of this serum pool by the complement-fixation method with infected and normal dog liver antigens consistently gave titers between 1:128 and 1:256 with the former, and no fixation whatsoever with the latter. *Complement-fixation test.* The technic used was essentially the Kolmer-Boerner test(7), which utilizes overnight icebox incubation. Infected tissue culture fluids were diluted in normal

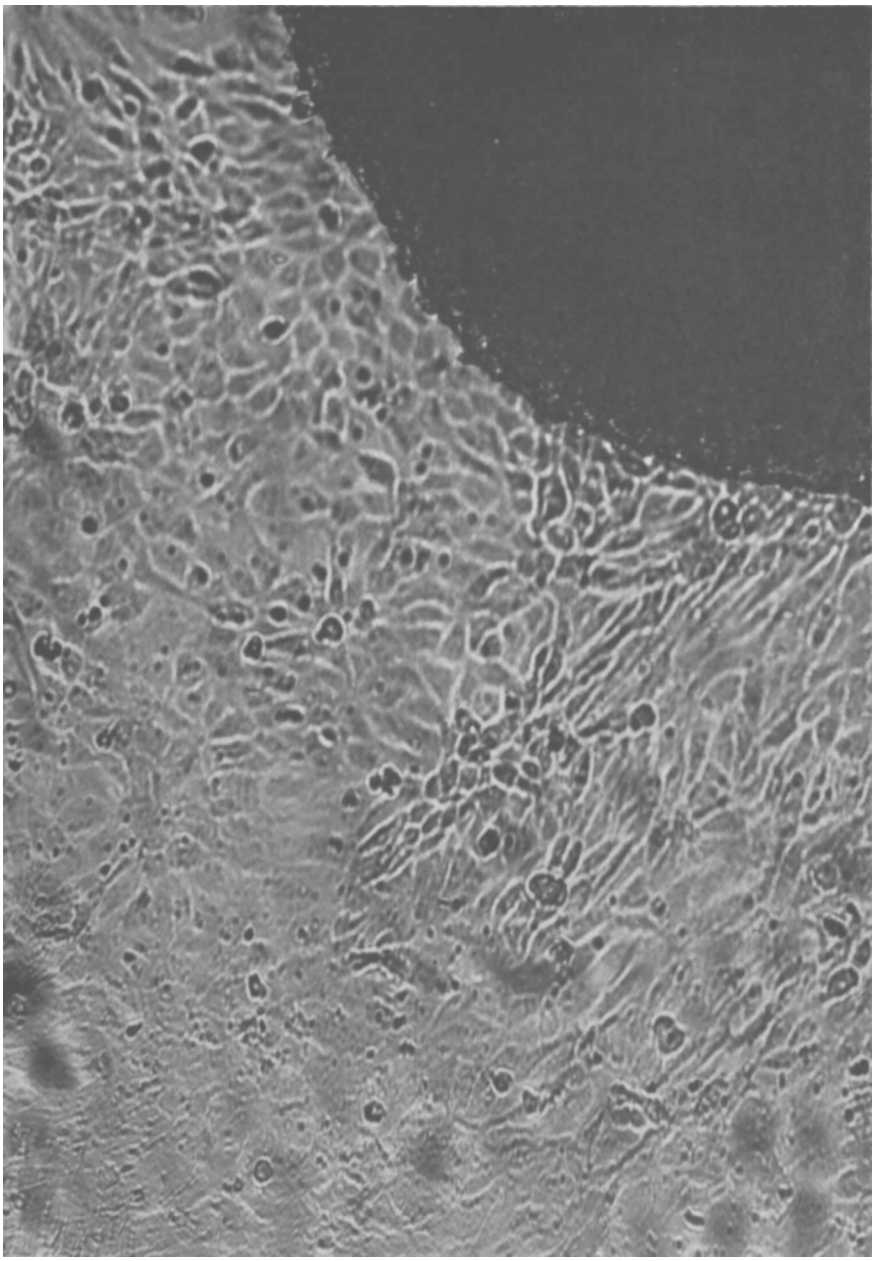

FIG. 2. Epithelial cell growth of monkey kidney tissue not infected with ICH virus. × 175.

saline in duplicate serial 2-fold dilution lines, from 1:4 to 1:128, and from 1:4 to 1:16. Addition of 8 units of immune serum to the first line of tubes and of a corresponding dilution of normal dog serum to the second followed. After distribution of 2 full units of complement, incubation was carried out at 4°C for about 18 hours. Two per cent sheep cells and 2 units of hemolysin were then added, and reading was made after further incubation for 15 minutes at 37°C.

*Experimental. Serial passage of infected dog liver in monkey, rabbit and dog kidney cultures.* The original inoculum of ICH virus,

which consisted of a 1:100 dilution of infected dog liver in glucosol solution(8) containing 50 units of penicillin and 50 mg of streptomycin per 1 ml. was introduced simultaneously into several tubes of monkey, rabbit or dog kidney tissue culture. Although no changes occurred in the monkey or rabbit kidney culture tubes, the fluids were harvested at the end of 7 days' incubation and those of each species pooled separately. In the dog kidney culture tubes a distinct cytopathogenic alteration in all tubes began on the fifth day following inoculation (Fig. 1), whereas control tubes remained normal throughout (Fig. 2). The infected dog kidney tubes were harvested and pooled on the sixth day. Second and third transfers were made with the 3 pools of fluid in homologous tissue cultures. Again no cytologic alterations were observed in either the monkey or the rabbit kidney series during the 7 days' incubation. However, beginning with the second passage in dog kidney culture, the cytopathogenic effect became evident on the second day and was complete on the fourth, when fluids were collected and pooled. Since the virus failed to induce any demonstrable change in monkey and rabbit kidney cultures for 3 consecutive transfers, the 2 series of passages were discontinued. However, 18 consecutive passages in the dog kidney culture series gave identical results: cytopathogenic effect started on the second day and was complete on the fourth, when fluids were routinely harvested.

*Serial passage of dog kidney culture virus in rabbit kidney and whole chick embryo cultures.* Passage of ICH virus in rabbit kidney cultures was attempted again, starting with harvest No. 2 of dog kidney culture. Seven consecutive transfers were made, each being harvested 7 days following inoculation. No cytopathogenic effect was observed in all 7 transfers, but assay for presence of virus which was made in dog kidney cultures at each passage level gave negative results. Passages in rabbit kidney cultures were again discontinued. A similar series was also made in whole chick embryo cultures with the sixth dog kidney passage harvest. Observation of the 6 serial transfers made in this tissue were impossible to interpret. The fibroblastic growth degenerated at the same rate in both infected and control culture tubes and masses of rounded cells began to appear on the third to fourth day. However, subcultures in dog kidney tissue at each level revealed the presence of virus in the second and third passages, but not in the fourth, fifth and sixth.

*Titration of dog kidney culture virus.* This was done on 4 occasions, with dog kidney harvests No. 4, 5, 10 and 15. Serial 10-fold dilutions were made with each in glucosol solution and inoculated into groups of 3 or 4 dog kidney culture tubes per dilution. Observation for 7 days revealed passage No. 4 to be completely cytopathogenic through the $10^{-4.0}$ dilution and only partially so at the $10^{-5}$. The cytopathogenic effect of passages 5, 10 and 15 was complete through the $10^{-5}$ dilution and partial at $10^{-6.0}$. In the 4 passage levels, a gradual delay of the cytopathogenic effect was observed from the $10^{-3.0}$ dilution upward.

*Comparison of ICH virus growth in normal and immune dog kidney cultures.* Unless they are bred and raised in strict isolation, it is a practical impossibility to trace adequately the past infection history of most puppies or dogs acquired for experimental purposes. It seemed essential, therefore, to determine whether kidney tissue from animals immune to ICH would be unfit for the propagation of the virus. Two pups, one 5 months old and susceptible to hepatitis, and the other 3 months of age and immune following recovery from experimental infection, were sacrificed on the same day. Tissue culture tubes were separately prepared with kidney tissue from each animal. Groups of tubes from each dog were inoculated with the sixth dog kidney culture harvest in dilutions ranging from undiluted to $10^{-3.0}$. Daily observation of the tubes showed no difference in the time of appearance of the cytopathogenic effect in tissues of either animal. Following this experiment, kidney tissue from immune animals has been routinely used with satisfactory results.

*Identification of Dog Kidney Culture Virus. Complement-fixation test.* This test was performed on 14 consecutive harvests of the virus in dog kidney cultures. The fluids were titrated as antigens in the presence of ICH im-

TABLE I. Antigenic Complement Fixing Titers of 14 Consecutive Passages of ICH Virus in Dog Kidney Cultures, in the Presence of ICH Immune Fox Serum.

| Passage No. | C.F. titer | Passage No. | C.F. titer |
|---|---|---|---|
| 1 | 16* | 8 | 32 |
| 2 | " | 9 | 16 |
| 3 | " | 10 | " |
| 4 | " | 11 | 32 |
| 5 | " | 12 | " |
| 6 | " | 13 | " |
| 7 | 32 | 14 | " |

* Reciprocal of highest dilution of infected fluid giving complete prevention of sheep cell hemolysis.

mune fox serum, as described under *Methods.* Results are summarized in Table I, where antigenic titers are expressed as the reciprocal of the highest dilution of fluid which completely prevented the hemolysis of sheep cells. Appreciable titers were obtained from the very first passage of the virus. A further 2-fold titer increase became evident on the 7th and 8th passages, and was uniformly obtained from the 11th to the 14th. No fixation whatsoever was obtained when the low dilutions of all fluids were tested in the presence of normal dog serum.

*Neutralization test.* Qualitative neutralization tests were made with harvests No. 3, 8, 9 and 13. Invariably, complete cytopathogenic effect was observed with the normal dog serum mixture by the end of the fourth day of incubation, whereas no changes whatever took place within 7 days with either of the immune fox serum mixtures.

*Titration of neutralizing antibodies in ICH fox immune serum.* Although the complement-fixing titer of the immune fox serum was known through repeated measurements, its neutralizing antibody content remained unknown since the only available procedure would have required a relatively large number of susceptible puppies. Those engaged in canine virus research are familiar with the difficulties involved in securing susceptible puppies for experimental purposes and in protecting them against natural infection with the agent under study. The ability of ICH virus to cause a demonstrable cytopathogenic effect in tissue culture afforded for the first time a practical method for the measurement of neutralizing antibodies. At first, the inactivated immune fox serum was diluted in glucosol in 4-fold steps, ranging from 1:4 to 1:1024. To each serum dilution an equal volume of a 1:500 dilution of the fifth harvest fluid was added, bringing the serum dilution range to 1:8 to 1:2048, and giving a final harvest fluid dilution of $10^{-3.0}$. As determined by a previous titration, the virus content at this dilution falls between 100 and 1,000 tissue culture infective doses. A mixture of equal volumes of inactivated, undiluted dog serum and 1:500 harvest fluid dilution served as control. All mixtures were incubated for one hour at 37°C, and each inoculated into 3 tissue culture tubes. Whereas the control mixture showed cytopathogenic effect starting on the third day and complete on the fifth, all immune serum-virus mixtures had no effect on the tissue culture growth in 5 days.

To determine the serum-antibody endpoint, a second test was made, identical to the first except for the serum dilution range, which extended in 2-fold steps from 1:512 to 1:8192. This time, complete protection of the tissue growth was observed through the 1:4096 dilution, while a cytopathogenic effect similar to that of the control mixture occurred at the 1:8192 dilution.

*Discussion.* Since the identification of ICH as a disease entity of the dog by Rubarth(9), a number of investigators have attempted the propagation of the virus outside its natural host range. Some successful efforts have been reported which, however, still remain unconfirmed. Thus, Miles and coworkers(10) passed the virus serially in the chick embryo for 12 transfers, and reproduced a mild case of the disease with the last chick embryo passage in one out of two dogs. Goret and others(11), claimed the probable transmission of dog liver virus to the ferret through nine passages. The disease induced in the ferret resembled distemper more and more with each passage, and became indistinguishable from it after a limited number of transfers. Later, Lucam and Goret(12) reported the serial propagation of the ferret-adapted agent in the embryonated hen's egg which resulted in a rapidly decreasing virulence for the ferret. Goret and others(13) reported also that the

FIG. 3. Experimental ferret showing opacification of right eye 2 to 3 wk after inj. of infected dog liver. Note clarity of left eye which received normal dog liver at same time.

experimental disease was reproducible only in ferrets of French origin, and not in those obtained from England. Furthermore, ferrets of French origin seemed to be susceptible only during a definite period of the year. Finally, Goret *et al.*(14) noted a febrile response in rabbits inoculated with infected dog liver, obtaining this response through 15 serial rabbit transfers. Except for a brief mention of the successful reproduction of the disease in susceptible puppies with the ferret-propagated virus(13), there were no attempts to demonstrate the identity of the chick embryo grown virus or of the agent responsible for the febrile response in rabbits either by serological means or by direct puppy inoculation.

Two of the present authors(15) tried repeatedly to propagate ICH virus in mice, hamsters, rabbits, distemper-immune ferrets, or chick embryos, using various routes of inoculation, with negative results. The susceptibility of the distemper-immune ferret was limited to the extent that, when infected dog liver suspension was injected intraocularly, a frank corneal opacification ensued (Fig. 3). This lasted for several months, with gradual clearing and return to normal. No such opacification occurred when a normal dog liver suspension was introduced into the second eye of the same animal. At no time were symptoms of generalized infection noted, even following simultaneous inoculations of large doses of infected dog liver suspension by the intracerebral and intraperitoneal routes. However, specific ICH complement-fixing antibodies were demonstrated in the sera of ferrets one and one-half and three months following inoculation of infected dog tissue by any route.

Although the present cultivation of ICH virus has not removed the agent from its normally susceptible host range, it represents a great advantage to the research worker over the use of live puppies. It constitutes a relatively simple and reliable method of virus assay which is being used advantageously in this laboratory in further attempts to adapt the virus to the chick embryo and other laboratory animals. It is also useful for obtaining accurate measurements of serum antibodies by the neutralization method.

A first practical application of the tissue culture virus has been the preparation of complement-fixing antigen by the present authors(15). Unlike the poliomyelitis antigen produced by a similar method by Svedmyr and others(16), which required a concentration of up to 600 x for use in the test, freshly harvested ICH-infected tissue culture fluids have titers high enough for use without concentration.

Of the two types of cells produced by dog kidney tissue—fibroblasts and epithelial cells —only the latter underwent cytopathogenic degeneration following virus infection. In cultures where both fibroblasts and epithelial cells grew side by side, only the epithelial cells were affected, whereas the neighboring fibroblasts retained their normal morphology. It was also observed that fibroblastic growth was more abundant with kidney tissue from younger than with that from older dogs. In one instance when embryonic dog kidney was used, almost a pure fibroblast culture was obtained with only rare epithelial cells. These embryonic cultures were found unsuitable for use in this study, since no cytopathogenic

effect became apparent even after seven days' incubation. However, by contrast, almost pure cultures of epithelial cells were obtained when the kidney tissue of puppies six months of age or older was used. As best as could be determined, satisfactory cultures were regularly produced with kidney tissue from pups at least 3 months old.

The effect of the tissue culture virus on susceptible puppies is being studied at the present time, and will be reported later.

*Summary.* The serial propagation of ICH virus by the roller tube method in dog kidney tissue culture through 15 consecutive passages is reported. The growth of the virus under these conditions causes a specific cytopathogenic degeneration of the tissue growth, similar to that reported for poliomyelitis virus in the presence of human or monkey tissues. Identification of the tissue culture virus was made by both the complement-fixation and serum neutralization methods using a known infectious canine hepatitis fox immune serum.

1. Weller, T. H., *New England J. Med.*, 1953, v249, 186.

2. Riordan, J. T., Ledinko, N., and Melnick, J. L., *Am. J. Hyg.*, 1952, v55, 339.

3. Syverton, J. T., and Scherer, W. F., *Bact. Proc.*, 1953, 42.

4. Coffin, D. L., and Cabasso, V. J., *Am. J. Vet. Res.*, 1953, v14, 254.

5. Coffin, D. L., Coons, A. H., and Cabasso, V. J., *J. Exp. Med.*, 1953, v98, 13.

6. Morgan, J. F., Morton, H. J., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.

7. Kolmer, J. A., and Boerner, F., *Approved Laboratory Technic*, ed. 3, D. Appleton Century Co., Inc., New York, 1941, 616.

8. Parker, R. C., *Methods of Tissue Culture*, ed. 2, Paul B. Hoeber, Inc., New York, 1950, 79.

9. Rubarth, S., *Acta Path. et Microbiol. Scand.*, Supp. 69, 1947.

10. Miles, J. A. R., Parry, H. B., Larin, N. M., and Platt, H., *Nature*, 1951, v168, 699.

11. Goret, P., Joubert, L., and Buffet, A., *Bull. Acad. Vet. de France*, 1950, v23, 305.

12. Lucam, F., and Goret, P., *Compt. rend. Acad. sc.*, 1951, v233, 277.

13. Goret, P., Lucam, F., Joubert, L., Flachat, Ch. and Henry, R., *Compt. rend. Soc. biol.*, Paris, 1952, v146, 1201.

14. Goret, P., Martin, L. A., and Joubert, L., *Bull. Acad. Vet. de France*, 1952, v25, 393.

15. Cabasso, V. J., and Stebbins, M. R., unpublished data.

16. Svedmyr, A., Enders, J. F., and Holloway, A., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 296.

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## Effect of 1,1-Dimethyl-4-Phenylpiperazinium Iodide on Peristaltic Reflexes of Isolated Guinea Pig Ileum. (20844)

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1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP) was shown to be a potent stimulant of the autonomic ganglia(1). The question has been raised as to whether or not this compound produces paralysis of the ganglia at high concentrations as does nicotine. The inquiry is of theoretical interest in studying the chemical transmission of nerve impulses, since the ganglionic stimulating and paralyzing actions of nicotine may be explained by depolarization and overdepolarization at the synapses induced by low and high concentrations respectively of the liberated acetylcholine(2). In our experiments on pentobarbitalized dogs, no difference in hypertensive response was noticed upon repeated intravenous injections of submaximal doses of DMPP (20-30 $\mu$g/kg). However, by previous administration of 0.75 mg/kg of N-(2-bromoethyl)-N-ethylnaphthalene methylamine.HBr (SY 28, an adrenergic blocking agent) in order to suppress partially the hypertensive effect of DMPP, there was produced some diminution in hypertensive response to the second injection of 0.1 mg/kg of DMPP 5 minutes later(3). These results seem to indi-