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Influence of Anticholinesterase Diisopropylfluorophosphate on Cardio-vascular and Respiratory Reflexes of Carotid Sinus Origin.

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In previous investigations we were able to show that the anticholinesterase prostigmine has no effect on the central transmission of the carotid sinus pressoceptive reflexes acting on the cardio-inhibitory, vasomotor and respiratory centers.[1] Recently O. Bodansky and A. Mazur[2] reported on the very marked anticholinesterase activity on blood and tissues of diisopropylfluorophosphate (D.F.P.).

The influence of the anticholinesterase D.F.P. on the respiratory, cardiac and the vasomotor reflexes induced by the carotid sinus presso-ceptors in the dog has been investigated.

Method. In the dog, under choralosane anesthesia, the efferent arteries of the carotid sinus were ligated, care being taken not to sever the pressoceptive innervation. The cephalic end of the common carotid artery was connected with a pressure device. By means of this technic, the hydrostatic pressure may be increased or decreased in the isolated presso-sensitive innervated carotid sinus. The cardio-vascular and respiratory reactions of the animal to the intracarotid sinus pressure changes were registered before and after injection of the anticholinesterase D.F.P. The cholinesterase activity of the blood was determined by means of the method described by A. L. Delaunois and H. Casier.[3]

Results. Intravenous injections of doses of D.F.P. (0.12-1 mg/kg) inhibiting completely the cholinesterase activity of the blood, do not stimulate the respiratory center and do not affect either the heart rate or the blood pressure. The cardio-vascular and respiratory reflexes induced by increase or decrease of the intracarotid sinus pressure are not affected by the administration of D.F.P. and the inhibition of the cholinesterase activity. The above results do not support the theory of a cholinergic transmission of the respiratory and cardio-vascular reflexes induced by means of a physiological stimulation of the carotid sinus pressoceptors.

[1] Heymans, C., Pannier, R., and Verbeke, R., *Thirtieth An. Meet. Fed. Am. Soc. f. exp. Biol.*, 1946.

[2] Bodansky, O., and Mazur, A., *Fed. Proc.*, 1946, **5**, 123.

[3] Delaunois, A. L., and Casier, H., *Experientia*, 1946, II, 2, 4.

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Growth of Influenza Virus in Eggs in the Presence of Bacterial Contamination and Streptomycin.*

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(Introduced by Chester S. Keefer).

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With a few possible exceptions viruses appear to be unaffected in their growth in living hosts in the presence of a wide variety of antibiotics.[1] In general one would ex-

* We are indebted to Dr. Chester S. Keefer for the supply of streptomycin, and to Miss Marjorie Jewell and Miss Carla Bryan for determining the streptomycin levels.

[1] Kramer, S. D., Geer, H. A., and Szobel, D. A., *J. Immunol.*, 1944, **49**, 273.

pect that the addition of a chemotherapeutic agent to virus-containing specimens with bacterial contamination would suppress bacterial growth without interfering with the proliferation of the virus. Although the influenza virus will grow readily in the allantoic or amniotic sac of the chick embryo in the presence of heavy bacterial contamination contained in unfiltered throat washings[2-4] the growth of virus is favored and the life of the embryo tends to be prolonged when penicillin is present.[3,5] Attempts to use zephiran[2,5,6] and sulfadiazine[5] as a means of controlling bacterial contamination in eggs were unsuccessful.

Streptomycin has been shown to have no observable adverse effect on the developing chick embryo or the proliferation of influenza virus in the allantoic sac.[7] It also prevents the growth of certain gram negative organisms in the chick embryo[8] and it has been shown to appear in the blood in small concentrations when applied to the surface of the chorioallantoic membrane.[9] In the present study, some of these observations have been confirmed and streptomycin has been further shown to be highly effective in preventing interference with the growth and recovery of influenza virus by the naturally occurring organisms present in unfiltered throat washings and feces.

Materials and Methods. Throat washings, stool suspensions and dilutions of virus were made with sterile brain broth containing 5% horse serum. Human group O cells were used in the virus titrations and for identification of virus by inhibition of red cell agglutination[10] with specific serum prepared in rabbits. All injections in eggs were made into the allantoic sac of 10- to 11-day white leghorn embryos with .1 cc volumes of various mixtures of streptomycin, contaminated material and virus. The eggs were incubated at 36°C to 38.5°C in a regular bacteriologic incubator. The PR8 strain of Influenza A virus and the Lee strain of Influenza B virus were diluted from infected allantoic fluids stored in sealed tubes at —60°C. As grown in this laboratory, the titer of Influenza A virus in the allantoic fluid after incubation for 48 hours, as measured by red cell agglutination,[10] was 1:64 to 1:128 and that for Influenza B virus was 1:8 to 1:16, occasionally 1:32. The streptomycin was prepared by Merck and Company (Lots No. 251 and 412) and was dissolved in distilled water to make a solution containing 100,000 units per cc. Assays of streptomycin in allantoic and amniotic fluids were carried out by an adaptation of the cup plate method in which a strain of staphylococcus was used as the test organism.[11]

Results. Injection of streptomycin and virus into the allantoic sac. Varying amounts of streptomycin were injected into the allantoic sac of chick embryos and, after 48 hours' incubation, a number of allantoic and amniotic fluids were assayed for streptomycin. The results are shown in Table I. Eggs No. 1 to 12 received streptomycin alone. All the embryos were alive at the end of 48 hours and had a normal appearance. Eggs No. 13 to 24 received a mixture of streptomycin and Influenza A virus in a final dilution of 10^{-7}. At the end of 48 hours the embryos were also alive and virus was present in each allantoic fluid in the usual titer. Eggs No. 25 to 36 were likewise injected with a mixture of streptomycin and Influenza B virus in a final dilution of 10^{-4}. All but 2 embryos (No. 25 and 32) were alive at the end of 48 hours and virus was again present in the usual titer.

[2] Eaton, M. D., Corey, M., van Herick, W., and Meiklejohn, G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 6.

[3] Hirst, G. K., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 155.

[4] Rickard, E. R., Thigpen, M., and Crowley, J. H., *J. Immunol.*, 1944, **49**, 263.

[5] Rose, H. M., Milloy, E., and O'Neill, E., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 23.

[6] Personnel of U. S. Naval Laboratory Research Unit No. 1, *Science*, 1942, **96**, 53.

[7] Florman, A. L., Weiss, A. B., and Council, F. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 16.

[8] Jones, D., Metzer, J. J., Schatz, A., and Waksman, S. A., *Science*, 1944, **100**, 103.

[9] Lee, H. F., Stavitsky, A. B., and Lee, M. P., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 143.

[10] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

[11] Stebbins, R. B., and Robinson, H. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 255.

TABLE I.
Concentration of Streptomycin in the Allantoic and Amniotic Fluids 48 Hours After Injection of Streptomycin into the Allantoic Sac.

Units of streptomycin injected into allantoic sac								
2,500			250			50		
	Units per cc after 48 hr in			Units per cc after 48 hr in			Units per cc after 48 hr in	
Egg No.	Allantoic fluid	Amniotic fluid	Egg No.	Allantoic fluid	Amniotic fluid	Egg No.	Allantoic fluid	Amniotic fluid
1	172	10	5	33, 46		9	4.4	±
2		5	6	>26	5	10		1
3	216	10	7	>26	5	11	8	±
4		10, 11	8	14		12		±
13	400	0	17	17	0	21	4.7	0
14	66, 88*		18	20	0	22	0	
15	66, 88		19	14, 20		23	4.5	
16	>52, 120	0	20			24	4.4	
25	160		29	10, 16	1.1	33	3.4	±
26	90		30	16		34	3	±
27	90, 122	4, 5	31	16	4	35	5	±
28	90	8, 10	32			36	4.5	±

* The presence of 2 figures in a square indicates that duplicate assays were made.

These results indicate that the injection of 2,500 units or less of streptomycin into the allantoic sac of the 10- to 11-day chick embryo is without apparent adverse effects on the embryo and does not interfere with proliferation of Influenza A or B virus. Furthermore, with the largest amount of streptomycin used, highly bacteriostatic or bactericidal concentrations persist in the allantoic fluid for at least 48 hours, but only small amounts appear in the amniotic fluid.

Experiments with Throat Washings. To throat washings obtained from a healthy adult were added Influenza A virus to make a final dilution of 10^{-8} and streptomycin to make a final concentration of 2,500 units per cc. After standing for an hour,† 1.5 cc was mixed with .5 cc of a solution containing 100,000 units of streptomycin per cc. Each of 6 10-day eggs was injected intra-allantoically with .1 cc of this mixture. A second group of 6 eggs was injected with throat washings and virus with no streptomycin added. After 48 hours' incubation, all 6 eggs receiving virus and throat washings without streptomycin were grossly contaminated and no attempt was made to demonstrate influenza virus. Of the 6 eggs receiving the mixture containing streptomycin, 2 were dead and 4 were alive, no organisms were seen on smear and none grew on culture. All fluids from these 6 eggs gave agglutination of red cells in a titer of 1:64 or higher.

Streptomycin and Suspensions of Stool. A mixture of human stool in a final dilution of 1:50, Influenza A virus in a final dilution of 10^{-7} and streptomycin in a final concentration of 2,500 units per cc was allowed to stand at room temperature for one hour, after first removing the coarser stool particles by spinning at 1,500 r.p.m. for 5 minutes. To 1.5 cc of this mixture was added .5 cc of a solution of streptomycin containing 100,000 units per cc and .1 cc was then injected into a number of eggs. Each egg received, therefore, slightly more than 2,500 units of streptomycin in combination with stool suspension in a final dilution of approximately 1:66 and virus. After incubation for 48 hours, the allantoic fluid was removed from those embryos showing no gross evidence of contamination as indicated by marked cloudiness of the fluid, foul odor and, frequently, rupture of the yolk sac.

† This was done on the assumption that the streptomycin would act more effectively on the contaminating organisms.

Preliminary tests with virus-stool mixtures in the absence of streptomycin yielded grossly contaminated embryos in every instance. This was also true of eggs receiving virus-stool mixtures containing 10,000 units of penicillin per cc. Of 36 eggs injected with a mixture containing stool, virus and streptomycin, 6 embryos were grossly contaminated and were discarded. Of the remaining 30 embryos, 23 were dead but the embryos appeared intact, there was no odor, and the allantoic fluid was only faintly cloudy. The fluid was indistinguishable in appearance from that obtained from embryos in which influenza virus has grown without added contaminating material. The allantoic fluid of the 6 living embryos had a similar appearance. Smears of these 30 fluids stained by the Gram method showed no organisms. However, culture on blood agar under aerobic conditions was positive in 6 instances. Five of these fluids were obtained from dead embryos. Influenza virus was readily demonstrable by red cell agglutination in each of the 30 fluids and the titers varied between 1:64 and 1:128. Control tests done with allantoic fluid derived from 6 eggs injected with mixtures of streptomycin and stool suspension without added virus failed to show any agglutination of red cells by such fluids. All embryos in this group were alive at the end of 48 hours.

Influenza A virus, free from any demonstrable bacterial contamination either by aerobic or anaerobic culture, was readily obtained by Seitz filtration of virus-infected allantoic fluid from an embryo receiving an injection of Influenza A virus, stool suspension and streptomycin. The filtered virus produced typical pulmonary lesions following intranasal instillation in mice, grew well in the allantoic sac and failed to cause agglutination of red cells when mixed with serum prepared against Influenza A virus, but did cause agglutination when mixed with serum prepared against Influenza B virus.

The results of these studies made with mixtures of streptomycin, suspensions of human stool and Influenza A virus indicate that virus will grow readily in the allantoic sacs of 10- to 11-day chick embryos in the presence of fecal contamination. It is doubtful that all such virus-infected fluids are entirely free from bacterial contamination, but Seitz filtration of the allantoic fluid yielded Influenza A virus free from detectable bacterial contamination and indistinguishable in its behavior from the original virus. Frequent death of the embryos was observed in these experiments which could not be attributed either to the virus alone or to streptomycin. The failure of a streptomycin-stool mixture, without added virus, to kill chick embryos suggests that death in the presence of virus is due to the combination of virus and fecal contamination.

Conclusions. 1. Streptomycin appears to have no adverse effect on the 10- to 11-day chick embryo when injected into the allantoic sac.

2. Streptomycin is demonstrable in bacteriostatic or bactericidal concentrations in the allantoic fluid 48 hours after a single injection of 2,500 units into the allantoic sac. Only small amounts appear in the amniotic fluid.

3. Streptomycin in the concentrations used appeared to have no adverse effect on the growth of either Influenza A or B virus in the allantoic sac.

4. Streptomycin is highly effective in preventing gross contamination of allantoic fluids of chick embryos injected with unfiltered throat washings and suspensions of human stool.

5. Influenza A virus, when highly diluted and mixed with unfiltered throat washings or stool suspensions containing added streptomycin, can be readily recovered after growth in the allantoic sac of the developing chick embryo.

6. The results indicate that the loss of virus which may follow removal of contaminants by filtration may be avoided by the addition of streptomycin to unfiltered specimens derived from animals or man. After growth in the chick embryo, the virus may then be recovered free from contamination by filtration if necessary.