INTRODUCTION OF ANTIVIRAL DRUGS INTO EGGS BY THE AIR SAC ROUTE

HENRY H. BLOOM[1] AND FRANCIS B. GORDON[2]

Camp Detrick, Frederick, Maryland

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Inoculation of the chick embryo for experimental purposes is accompanied by a certain probability of death of the embryo due to the trauma of the inoculation. Although the incidence of such deaths from a single inoculation may be sufficiently low to be insignificant, when repeated injections are made into the same egg, the number of traumatic deaths that occur may be great enough to interfere with the interpretation of the experiment.

This problem was encountered when groups of 8 day eggs were inoculated initially with psittacosis virus into the yolk sac, followed by three inoculations of penicillin into the allantoic cavity, the first immediately following the virus and the others on the fifth and seventh days thereafter. Evidence was obtained that nonspecific deaths caused by the repeated inoculations, and occurring during the period of specific viral deaths, falsely increased the apparent virus titer by at least 1 log unit.

The study of this problem resulted in the discovery that satisfactory concentration within the egg can be achieved by introducing the drug into the air sac and allowing it to diffuse through the inner layer of the shell membrane. This method has the advantage of simplicity, less trauma to the egg, and less chance of introducing contamination. Two procedures were followed to evaluate this method of drug administration compared with direct inoculation into the allantoic cavity:

(1) Tests were performed in virus infected eggs in which the protective effects of drugs administered by the two methods were observed.

(2) Biological assays of penicillin present in allantoic fluid were performed at various time intervals following administration of the drug by the two methods.

[1] Present address: Fleet Epidemic Disease Control Unit No. 2, Fleet Post Office, San Francisco, California.

[2] Present address: Naval Medical Research Institute, Bethesda, Maryland.

MATERIALS AND METHODS

Virus. The 6 BC strain of psittacosis virus was used. It has been maintained in this laboratory for some years as egg passage material stored at the temperature of dry ice. For this series of tests a 10 per cent suspension of infected yolk sac tissue was prepared in nutrient broth. Following light centrifugation the supernate was dispensed in small quantities into vials which were sealed in a flame. After shell-freezing, the preparation was kept in a dry ice chest until used. The titer of the virus used in these experiments was $10^{8.6}$ egg LD_{50}'s per 0.25 ml.

Drugs. The drugs were prepared in sterile distilled water, and 0.25 ml was inoculated. The aureomycin solution, containing 0.5 mg per 0.25 ml, was made fresh each day. Sodium sulfadiazine was used in a concentration of 5.0 mg per 0.25 ml and was passed through a sintered glass filter to insure a preparation free of bacteria. Sodium G penicillin was used in various concentrations as indicated.

Penicillin assay. The horizontal diffusion method was used for the biological assay of penicillin. Stainless steel assay cups of 5.7 mm inside diameter with a capacity of 1 ml were used to hold the material to be assayed. The double layer pre-seeded agar plate method of Schmidt and Moyer (1944) was utilized. This consisted of pouring 15 ml of nutrient agar into a 4-inch, flat-bottomed petri dish and allowing the agar to solidify. Nutrient agar was cooled to 48 C, and a 1 per cent seeded agar was prepared from a 24 hour shake culture of *Micrococcus pyogenes* var. *aureus* strain no. 209, the test organism. Three ml of this seeded agar were poured on top of the first layer of agar, and the stainless steel assay cups were placed in position after a 30 minute hardening period. These cups were placed with the beveled end down and were allowed to settle by their own weight on the agar surface.

Dilutions of the various components of the egg to be assayed were first prepared in a phos-

phate buffer, pH 6.8. Comparative tests indicated that no significant differences resulted when the egg materials were diluted in sterile distilled water so this became the diluent of choice.

The assay cups were filled almost to the top by the use of a syringe and needle. The plates were incubated at 37 C for 16 to 20 hours, after which the zone of inhibition produced around each cup was measured, with a millimeter rule, with the plate in an illuminated colony counter.

Fresh penicillin standards were included with each day's assay. Solutions of penicillin, containing 0.5, 1.0, 2.0, and 4.0 units per ml were used as standards to plot the standard curve. The 1 unit standard was included on all plates, standard and unknown, to help correct for plate to plate variation. The zones of inhibition were measured and then corrected to the 1 unit standard. When the average zone of inhibition for each standard was plotted against the concentration of penicillin on 2 cycle semilog paper, the points fell along a straight line.

In all cases at least 2 dilutions of the unknown were assayed in duplicate and the results averaged. No results were used where the zone of inhibition produced was significantly greater than the size of the zone produced by the 4 unit standard.

Allantoic fluid. One ml portions of allantoic fluid obtained by aspiration from each of 6 eggs were pooled, and 1 ml of the pool was used for assay. Some difficulty was encountered with 9 day embryos since it was not always possible to obtain blood-free allantoic fluid from embryos of this age. But in most instances clear fluid was collected from the older embryos.

Virus inoculation. Twenty-five hundredths ml of the appropriate dilution was inoculated into the yolk sacs of 8 day old chick embryos. The eggs, placed with the air sac end uppermost, were inoculated by the use of 1 inch 22 gauge needles inserted directly downward through the air sac and into the yolk sac.

Drug inoculations. These were performed also with the egg in the vertical position. Twenty-five hundredths ml was inoculated either into the allantoic cavity or into the air sac. Allantoic cavity inoculations were made with a 22 gauge, 1 inch needle inserted through the air sac. To administer drug by the air sac route the needle was directed just through the drilled portion of the shell and the inoculum expelled there. When inoculations of virus and drug were performed on the same lot of eggs, the virus injections were made first and were followed immediately by the drug.

RESULTS

Comparative protection test; daily inoculations of penicillin. A series of 10-fold virus dilutions, 10^{-4} through 10^{-9}, were each inoculated into the yolk sacs of 45 eight day embryos. Two thousand units of penicillin, contained in 0.25 ml, were administered immediately following virus inoculation and at 24 hour intervals thereafter for a 10 day period. In 15 eggs of each dilution group, the drug was injected into the allantoic cavity, and in 15, into the air sac. The remaining 15 eggs (controls) of each group did not receive

TABLE 1

Effect of daily inoculation of penicillin (2,000 units) by two different methods in psittacosis infected eggs

Dilution of Virus	Day of Death										Accumulated Dead/Total
	1	2	3	4	5	6	7	8	9	10	
No penicillin (control)											
10^{-4}	1	1		16	12						28/28
10^{-5}	2			4	23	1					28/28
10^{-6}	1			10	16	3					29/29
10^{-7}	1			1	19	8					28/29
10^{-8}	4					3	11	8	2	1	25/26
10^{-9}	3	1				1	4	3	1	1	10/26
Penicillin inoculated into allantoic cavity											
10^{-4}	5	3		1		2	3		3	1	10/22
10^{-5}	1	1	1	2	3	1	2	1	1	2	12/27
10^{-6}	2			1		1	1			2	5/28
10^{-7}	4	2		3		5				1	9/24
10^{-8}		2	1	1	1	1					3/27
10^{-9}	1			1		1			3	1	6/29
Penicillin inoculated into air sac											
10^{-4}				1				4		2	7/30
10^{-5}										1	1/30
10^{-6}	3		1								0/26
10^{-7}	5			2							2/25
10^{-8}	2	1		1					1		2/27
10^{-9}	1					1				2	3/29

Eggs dying in the first three days are not included in the final mortality score.

any penicillin. Two such experiments were performed which are lumped together in table 1.

Mortality rates for the various groups were calculated on the assumption that all deaths in the first three days were nonspecific and all others were specific. This assumption appears to be ordinarily valid with these virus dilutions as seen by inspection of the first portion (no drug control) of table 1. In both drug treated groups a high degree of protection from the virus is evident, but the pattern of deaths in the eggs receiving daily injections into the allantoic cavity, admittedly a severe procedure, suggests that many deaths throughout the period of observations were due to nonspecific cause, i.e., the trauma of repeated injection. Relatively small numbers of deaths occurred in the group of eggs that received penicillin into the air sac. Evidence presented below indicates that the two groups of eggs contained essentially the same amounts of penicillin.

Comparative protection test; single inoculations of penicillin. Approximately 10,000 LD_{50}'s of psittacosis virus were inoculated into the yolk sacs of a group of 8 day old embryos. Immediately following the virus injection a single inoculation of 1,000, 10,000, 20,000, and 50,000 units of penicillin was administered to each of 4 sets of eggs; one series was given the drug into the allantoic cavity, and the other, into the air sac.

TABLE 2

Protective effect of single injections of penicillin administered to psittacosis infected eggs by two different methods

Amount of Penicillin	Route of Drug Administration	Dead/Total
units		
1,000	Allantoic cavity	15/15
	Air sac	12/14
10,000	Allantoic cavity	7/14
	Air sac	8/15
20,000	Allantoic cavity	9/12
	Air sac	5/14
50,000	Allantoic cavity	2/11
	Air sac	3/14
None	—	15/15

10,000 LD_{50}'s of virus used per egg.

TABLE 3

Protection of psittacosis infected eggs by single inoculations of drugs using two routes of administration

Route of Administration	Drug	Day of Death									Mortality Rate
		1	2	3	4	5	6	7	8	9	10	
Air sac	Water (control)			1	7	21	1					29/29
	Sulfadiazine, 5.0 mg	1					14	1				15/29
	Aureomycin, 0.5 mg	1			2				1		4	7/29
Allantoic cavity	Water (control)	2			11	15	1					27/28
	Sulfadiazine, 5.0 mg	4				2	9	3	2	1	1	18/26
	Aureomycin, 0.5 mg	1					1	1		3	2	7/29

5,000 LD_{50}'s of virus used per egg.

One set of eggs which served as the control for the experiment did not receive any penicillin.

As indicated in table 2, for each drug level comparable results were obtained for both methods of drug inoculation.

Comparative protection test; single inoculations of aureomycin or sulfadiazine. Two experiments were performed to determine if the air sac route of drug administration was an efficient method for the introduction of aureomycin and sulfadiazine into psittacosis infected embryos. A group of 8 day old embryos was inoculated with approximately 50,000 LD_{50}'s of psittacosis virus. To one group of 30 eggs, 5.0 mg of sodium sulfadiazine were inoculated by the allantoic cavity route. Another group received the same dosage by the air sac route. Other groups received 0.5 mg of aureomycin by the two routes. Control groups received 0.25 ml of sterile distilled water by each of the two routes of inoculation (table 3).

On the basis of the comparative protection tests performed, it appears that there are no differences in the protection induced in psittacosis infected embryos by inoculating penicillin, aureomycin, or sodium sulfadiazine by the air

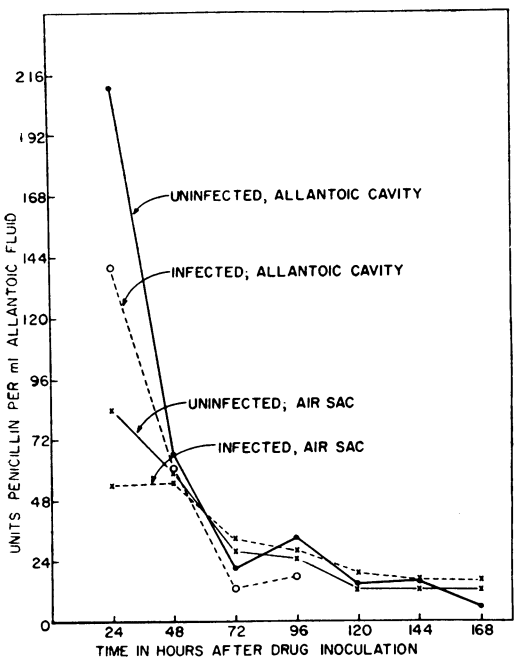

Figure 1. Assay of allantoic fluid for penicillin in uninfected and psittacosis infected eggs. Allantoic cavity or air sac used for inoculation of 1,000 units penicillin at time 0. Each point on the graph represents from one to six determinations.

sac route of administration rather than directly into the allantoic cavity.

Assay for penicillin. To further compare the two routes of drug administration, assays of allantoic fluid for penicillin were performed after injection of the drug by the two methods. Uninfected and psittacosis infected 8 day old embryos were each inoculated with 1,000 units of penicillin, either by the air sac or allantoic cavity route of inoculation. At each 24 hour interval thereafter 6 eggs of each series were opened. One ml of allantoic fluid was harvested from each egg to form a pool of which 1 ml was used for assay of penicillin. All results are reported as units of penicillin per ml of allantoic fluid. Values from several experiments were averaged and are graphically presented in figure 1.

The concentration of penicillin present in the allantoic fluid at 24 hours was significantly greater in the eggs, uninfected or infected, that received penicillin directly into the allantoic cavity. Also, the drug level at 24 hours was higher in uninfected eggs than in infected, whichever method of inoculation was used. The reason for this difference, if significant, is not apparent. At 48 hours and thereafter the drug level for all groups of eggs was similar.

DISCUSSION

It is apparent from the results presented that the air sac route is an efficient method of introducing certain drugs into the developing chick embryo, over a period of time, without substantially increasing the number of traumatic deaths.

No information is available on the mechanism by which these drugs reach the allantoic cavity after deposition in the air sac. To what extent they diffuse directly through the chorioallantoic membrane or are absorbed by the vascular system in the membrane and later excreted into the allantoic fluid is not known. Either mechanism is compatible with the observation of a lesser concentration of penicillin in the allantoic fluid at 24 hours when administered by the air sac route. The drugs presumably reach the yolk sac in similar concentration by the two methods since that tissue is heavily attacked by the viral infection, but no assays were performed on yolk or yolk sac. In a series of assays for aureomycin in the egg, following injection in the allantoic cavity, Allen and his colleagues (1953) found similar decreasing concentrations, but yolk sac was not included in their observations.

This technique may find use for tests with other compounds. It might be expected to be less effective with compounds of greater molecular size, but this has not been tested directly. The observations of Salvatori (1936) indicate that crystalloids, but not colloidal suspensions, would be expected to pass into the egg from the air sac. Where it is feasible, the technique reported here has the advantage of simplicity over that described by Collier (1951) in which a glass cannula, inserted into the yolk sac, remains in position throughout the experiment.

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SUMMARY

A comparison was made between two methods of administering drugs to developing chick embryos, i.e., into the allantoic cavity and into the air sac. Comparative protection tests in psittacosis infected eggs, employing penicillin, aureo-

mycin, and sodium sulfadiazine, indicated the same degree of protection obtained regardless of the route of drug inoculation. Biological assays of allantoic fluid, at 48 hours and later after penicillin inoculation, revealed no difference in concentration of drug as a result of administration by the two different routes.

Introduction of drug by the air sac route has several theoretical advantages over direct injection into the egg, especially when repeated injections are desired. The main advantage, demonstrated here in eggs to which drug was administered daily, is the reduction in numbers of nonspecific deaths due to trauma.

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