ous class of tissues. Incidental observations on *Amblystoma punctatum* demonstrated that the number of pycnotic nuclei and metaphase figures were not the same in the frog and salamander. Yet, if one considers the pycnotic nuclei as former metaphases which have become agglutinated, and the pseudo resting stages as transitionals from the colchicine metaphase figure to the pycnotic nucleus, then the sum total of all 3, in each species, is about equal. Then, the reaction to colchicine is qualitatively and quantitatively the same in both the frog and the salamander, both microscopically and grossly.

Summary and Conclusions. Colchicine was used in concentrations ranging from 1:1000 to 1:1,000,000. It was found to act as a capillary poison, to prevent ovulation when injected into sexually-mature female frogs prior to anterior pituitary stimulation, to prevent cleavage of normal eggs exposed to spermatozoa previously treated with colchicine, to affect gastrulation to such an extent that exo-gastrulae were the rule, to inhibit the appearance of organ anlage when applied just before the normal appearance time, to retard general growth of the larvae to such an extent that cephalic structures were markedly stunted and hatching prevented. The cytological picture was one of many pycnotic nuclei, occasional multinucleated cells, few metaphase figures, and apparent resting stages of mitosis.

15624

Simultaneous Cultivation of Typhus Rickettsiae and Influenza Virus in the Developing Chick Embryo.

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Over the past few years, large numbers of developing chick embryos have been used in this laboratory for the production of vaccines prepared from typhus fever rickettsiae and from influenza virus. In the preparation of typhus vaccine, the rickettsiae are cultivated in, and harvested from the yolk sac, whereas for influenza vaccine, the influenza virus is harvested from the allantoic fluid. In each instance, the remainder of the egg-contents is discarded. Thus, the allantoic fluid of the typhus eggs and the yolk sacs of the influenza eggs are wasted in the preparation of typhus vaccine and influenza vaccine, respectively. It had been observed, however, that in eggs inoculated with typhus rickettsiae in dilutions that did not result in the early death of the embryo, very few rickettsiae were found in the allantoic membranes. Moreover, it did not seem likely that rickettsiae, even if present, would be adsorbed on and subsequently eluted from fowl red blood cells, after the manner of the influenza virus.[1] Conversely, the adapted form of influenza virus used as seed material for vaccine production does not proliferate to any extent in the yolk sac.[2] In each instance, then, the portion of the egg that is discarded could be salvaged and used to advantage if both agents could be grown simultaneously in the same egg. The present communication records the results of a series of experiments in which vaccines were prepared from eggs inoculated with both typhus rickettsiae and influenza virus.

In preliminary experiments, it was found that an egg previously infected with typhus could actually be reinfected with influenza. In order to obtain a good yield of influenza, it would be essential, however, to regulate

[1] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 195.

[2] Beveridge, W. I. B., Burnet, F. M., and Williams, S. E., *Australian J. Exp. Biol. and Med. Sc.*, 1944, **22**, 1.

very carefully the dose of typhus. Accordingly, from the results of egg titrations[3] carried out on each batch of typhus seed material, a dose was selected that would produce an infection sufficiently heavy to kill most of the embryos on the 7th, 8th and 9th day following inoculation. Thus, the typhus-infected eggs could be reinoculated with influenza virus on the 5th day following the original rickettsial inoculation, and so allow the proliferation of the influenza virus for 2, 3 or 4 days before the death of the embryo.

Materials and Methods. Experiments were planned according to the following schedule.

A. Double infection of developing chick embryos with:

1. Epidemic strain of typhus rickettsiae and influenza virus A.
2. Epidemic strain of typhus rickettsiae and influenza virus B.
3. Murine strain of typhus rickettsiae and influenza virus A.
4. Murine strain of typhus rickettsiae and influenza virus B.

B. Vaccine preparation from eggs infected with both typhus and influenza:

1. Typhus vaccines from yolk sacs.
2. Influenza vaccines from allantoic fluid.

Eggs. White Leghorn eggs that had been incubated for 7 days were supplied by a dealer.

Seed Materials. All seed materials were derived from the stocks used in routine vaccine production.

For rickettsial inoculations, the Breinl strain of epidemic typhus and the Castaneda strain of endemic (murine) typhus were employed. For influenza virus inoculations, the PR8 strain of influenza A and the Lee strain of influenza B were used.

Inoculations and Incubation. Eggs that had been incubated for 7 days were candled in the upright position and the lower borders of the vascular membrane were indicated on the shell. The typhus rickettsiae were inoculated directly into yolk from the side of the egg and below the vascular area. After 5 days' further incubation, at 35°C, the eggs were candled and the dead embryos were discarded. Influenza virus was then introduced into the allantoic space of the surviving eggs, after which they were returned to the incubator, at 35°C. Thereafter, they were candled twice daily, and the dead embryos were saved for collection of yolk sacs and allantoic fluid.

Harvesting. During the course of the experiments, eggs were harvested and examined individually. The clear allantoic fluid was first aspirated and stored, and a small sample was kept for testing. At time of harvesting, the presence or absence of influenza virus in each sample was determined by examining for its ability to agglutinate fowl red blood cells.[4,5] The yolk sac was then separated, stripped free of yolk and placed in a vial. A small portion of each yolk sac membrane was used to make an impression smear that was stained with methyl violet and examined for the presence of rickettsiae. Allantoic fluids were stored in vials in the refrigerator at 4°C; yolk sacs were stored in vials in a CO_2 ice-box.

Preparation of Influenza Vaccine. Following the individual harvesting of each egg and examination of the samples, the allantoic fluids were combined so that fluids from eggs harvested on the same day were pooled together. Hemagglutination titers[6] were determined for each pool. After this estimation, all the pools were combined and were used for the preparation of a vaccine.[7] The vaccine was prepared by adsorbing the virus on fowl red blood cells in the cold, separating the supernatant fluid from the agglutinated cell mass and eluting the virus from the latter into one-tenth volume of saline at 37°C. To each vaccine was added a final concentration of 0.05% formalin and 0.01% merthiolate.

[3] Craigie, J., McClelland, L., and Parker, R. C., Memorandum No. 5 (Project Med. 8). Submitted to the Assoc. Comm. Med. Research, Nat. Research Council Can., May, 1943. (Withheld from publication for security reasons.)

[4] Hirst, G. K., *Science*, 1941, **94**, 22.

[5] McClelland, L., and Hare, R., *Canad. Pub. Health J.*, 1941, **32**, 530.

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

[7] Francis, T., and Salk, J. E., *Science*, 1942, **96**, 499.

TABLE I.
Protocol of a Typical Experiment.

Exp. No. 3 No. of eggs: 24	Date: Aug. 30, '45	Temp. of incubator: 35°C

Age of embryos at time of inoculation with rickettsiæ: 7 days
Strain and dose of rickettsiæ: Epidemic, Breinl (56E. 27.3.45); 0.2 ml of a 2×10^{-5} dilution of seed yolk sac per egg
Site of inoculation: side of egg, below vascular area, into yolk
Survivors after 5 days: 20 eggs (4 discarded)

Age of embryos at time of inoculation with influenza virus: 12 days
Strain and dose of influenza virus: B, Lee (Francis) E_3; 0.2 ml of a 10^{-2} dilution of seed allantoic fluid per egg
Site of inoculation: side of egg, into allantoic space.

TABLE II.
Results of Inoculations of Developing Chick Embryos with Typhus Rickettsiæ and Influenza Virus.*

Days after inoculation					
With typhus	With influenza	No. of dead eggs	Egg No.	Agglutination with fowl red blood cells (for influenza)	Yolk sac smear (for typhus)
7	2	7	1	positive	+++
			2	"	++
			3	"	++++
			4	"	+++
			5	"	+++
			6	"	+++
			7	contaminated	
8	3	6	8	positive	+++
			9	no fluid obt'd	++++
			10	positive	+++
			11	"	++++
			12	"	+++
			13	"	++++
9	4	7	14	"	++++
			15	"	+++
			16	"	++++
			17	"	++++
			18	"	+++
			19	"	+++
			20	"	+++

Hemagglutination titer of allantoic fluids:
(a) pooled on 2nd day after inoculation with influenza: 1/192
(b) pooled on 3rd day after inoculation with influenza: 1/256
(c) pooled on 4th day after inoculation with influenza: 1/192
Hemagglutination titer of finished influenza vaccine: 1/2000
* See Table I.

Preparation of Typhus Vaccine. The frozen yolk sacs were allowed to thaw, were pooled and were fragmented by shaking with glass beads. Saline containing 0.75% formalin was added to the pooled yolk sacs to make a 15% suspension by weight and to kill the rickettsiae. After 3 days at 4°C, the yolk sac emulsion was processed by treatment with ethyl ether according to the method[3] used in this laboratory for the preparation of typhus vaccine.

Experiments and Results. The protocol of a typical experiment is shown in Table I. In Table II are shown the results of the inoculations.

Testing for Antigenicity. Four influenza and 4 typhus vaccines were submitted for antigenic testing. Determinations of anti-

genicity were carried out by those* who were responsible for the routine antigenicity testing in the influenza and typhus vaccine units.

The 4 influenza vaccines tested (2 influenza A vaccines and 2 influenza B vaccines) were antigenic in mice and passed the requirements established by the U. S. Public Health Service, Division of Biologics Control as used in this laboratory in the antigenic testing of influenza vaccines.[8]

The 4 typhus vaccines tested (2 epidemic typhus vaccines and 2 murine typhus vaccines) were antigenic in mice and protected them against challenge doses of the toxic material regularly employed in this laboratory for the antigenic testing of typhus vaccines.[9]

Testing for Cross Immunity. Half of the mice that had been inoculated with influenza A vaccine and that had survived the challenge dose of influenza A virus were challenged, after a suitable interval, with epidemic typhus; the other half were challenged with murine typhus. This manner of cross challenging was repeated with each group of vaccinated mice that survived its own particular challenge material. Minimal doses of the heterologous challenge material were administered in each instance in order to detect any evidence of cross protection.

The groups of mice that had been protected with influenza vaccine, and that had survived the challenging dose of influenza virus, showed no evidence of immunity to either the epidemic or the murine strain of typhus rickettsiae.

In the groups receiving typhus vaccine, most of the mice that had survived the challenging dose of typhus, died following a challenge with influenza virus. The few surviving mice were sacrificed on the 10th day after the administration of influenza virus. All showed evidence of having been infected with influenza, as typified by lung consolidation ranging from 1 to 4+.

Summary and Conclusions. (1) Developing chick embryos were inoculated with rickettsiae of typhus fever, following which the same eggs were reinoculated with the virus of influenza. Two strains of rickettsiae were employed, namely, the Breinl strain of epidemic typhus and the Castaneda strain of murine (endemic) typhus. Also, 2 strains of influenza virus were employed, namely, the PR8 strain of influenza A and the Lee strain of influenza B. The groups of developing chick embryos inoculated with typhus and influenza, included the 4 possible combinations of rickettsiae and virus, *viz.* epidemic typhus and influenza A, epidemic typhus and influenza B, murine typhus and influenza A, and murine typhus and influenza B.

(2) Rickettsiae, inoculated into the yolk sac, were recovered from the yolk sac membranes, and typhus vaccines were prepared therefrom.

(3) Influenza virus, inoculated into the allantoic cavity, was recovered from the allantoic fluid and influenza vaccines were prepared therefrom.

(4) All the vaccines passed the requirements established for typhus and influenza vaccines produced in this laboratory.

(5) There was no evidence of immunity to influenza in the mice inoculated with typhus vaccine, and conversely, there was no evidence of immunity to typhus in the mice receiving immunizing doses of influenza vaccine.

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[8] Hare, R., and Mackenzie, D. M., in preparation.

[9] Craigie, J., in preparation.