chloro-4, 4′-diaminodiphenyl sulfone, like thromboplastin with Ac-globulin, activates prothrombin more rapidly and the yield of thrombin is increased. The similarity between the action of the artificial activators and the physiological ones would seem to be of more than coincidental significance, even though the time intervals involve hours in one instance and minutes in the other.

Perhaps the most remarkable result of these experiments is the difference in action of 3, 4, 4′-triaminodiphenyl sulfone and 3-chloro-4, 4′-diaminodiphenyl sulfone. One has an amino group in the three position and the other a chlorine atom. One is an inhibitor and the other an accelerator. Thus, for one set of conditions, the difference between a "coagulant" and an "anticoagulant" has been reduced to a locus on the benzene ring where placement of an amino group produces one effect and a chlorine atom the opposite. This subtle detail is easily brought into focus, and perhaps it is here where we can hope to see, for the first time, what forces physiological anticoagulants and coagulants bring to bear on prothrombin.

Summary. Purified prothrombin can be activated by dissolving it in a 25 per cent solution of sodium citrate, but the yield of thrombin is usually low. By adding a small amount of 3-chloro-4, 4′-diaminodiphenyl sulfone to the activation mixture the yield of thrombin is increased to the maximum. Several other diphenyl sulfones also increase the yield. In contrast, 3, 4, 4′-triaminodiphenyl sulfone and 2-hydroxy-4, 4′-diaminodiphenyl sulfone act as inhibitors of the activation process.

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Cultivation of Influenza Virus in the Chorio-Allantoic Membrane of Deembryonated Eggs.* (17541)

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Fertilized chicken eggs have proved to be an excellent medium for the growth of many viruses and other pathogenic agents. A number of viruses grow in the various tissues of the embryo and in the membranes enveloping it in an indiscriminate manner while others show specific affinities for certain structures in the egg. For a number of viruses, among them those of the influenza group, the chorio-allantoic membrane(1-3) is the main site of virus multiplication. Most other structures offer less favorable conditions. It seemed an obvious step, therefore, to use only the chorio-allantoic membrane *in situ* for virus growth and to eliminate the other constituents of the egg. For this purpose the following technic was found practicable.

Methods. Eggs which have been incubated for 14-15 days are candled and the outline of the air-sac is marked. One-half centimeter above this line the egg shell is cut with an electric drill and the piece of shell is removed. The free-lying shell membrane and the marginal zone of the shell both on the inside and outside along this opening are covered with hot paraffin. The shell membrane together with the piece of underlying chorio-allantoic membrane is then cut away with a pair of curved scissors. By gently tilting and rotating the egg the embryo and the yolk sac are poured out and their connections with the chorio-allantoic membrane are severed with

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1. Burnet, F. M., *Med. J. Australia,* 1935, v2, 687.

2. Nigg, C., Wilson, D. E., and Crowley, J. H., *Am. J. Hyg.,* 1941, v34, Sect. B, 138.

3. Pearson, H. E., *J. Bact.,* 1944, v48, 309.

TABLE I.
50% Endpoints of Virus Titrations Done in:

Exp. No.	(a) 10-11-day eggs allantoically	(b) in 15-day "empty" eggs	(c) difference in logs
1	$10^{-7.46}$	$10^{-10.00}$	2.54
2	$10^{-7.57}$	$10^{-9.0}$	1.43
3	$10^{-7.33}$	$10^{-8.57}$	1.24
4	$10^{-7.66}$	$10^{-8.71}$	1.05
		Avg diff.	1.57

scissors. The chorio-allantoic membrane which is firmly attached to the shell membrane is the only structure remaining in the egg. The internal surface of the membrane is now thoroughly washed with 3 changes of cold 0.85% NaCl so as to remove any remaining yolk, albumen, and blood. After the egg is drained by placing it with the open end down on a sterile petri dish, it is filled with 10-40 cc of Tyrode's solution containing virus, and 10 units of penicillin and 40 μg of streptomycin per cc. The opening is closed with a sterile rubber cap, such as is used for centrifuge tubes, which is lined and then sealed to the shell with hot paraffin. The eggs in suitable trays are then put in a test tube roller which is kept in an incubator at 37° and makes six revolutions per hour. Fluid can be withdrawn from the eggs at desired intervals by piercing the rubber cap with a sterile needle attached to a syringe.

It has also been found possible to infect eggs first allantoically in the regular way and after the virus has been adsorbed to the membrane or as late as 48 hours after infection the contents of the egg are poured out. The interior is washed thoroughly and filled with Tyrode's solution. After further incubation at 37° virus increase can be demonstrated.

Results. Thus far, the PR8 strain of influenza virus has been used in the experiments. Hemagglutination titers of 1280-2560 in a pattern test were regularly obtained in the fluid after 24-hours' incubation when a 10-20 ml volume of 10^{-5} to 10^{-6} dilution of virus was used as inoculum. Under the same conditions a hemagglutination titer of 5120 is usually obtained with allantoic fluid of the complete egg infected with the PR8 strain. The specificity of the reaction is demonstrated by the inhibition obtained with specific serum against influenza virus, Type A. It is obvious, therefore, that the chorioallantoic membrane produces virus under the described conditions in much the same amounts as in the intact egg. Several tests were done in which the sensitivity of this method for detecting small amounts of virus was compared with that of allantoic inoculation. Ten-fold dilutions of allantoic fluid infected with PR8 strain were prepared in Tyrode's solution and inoculated in 0.1 ml amounts in 10-11 day old eggs allantoically and in 20 ml amounts in egg cavities prepared by the method described. Four eggs were used per virus dilution. The allantoic inoculations

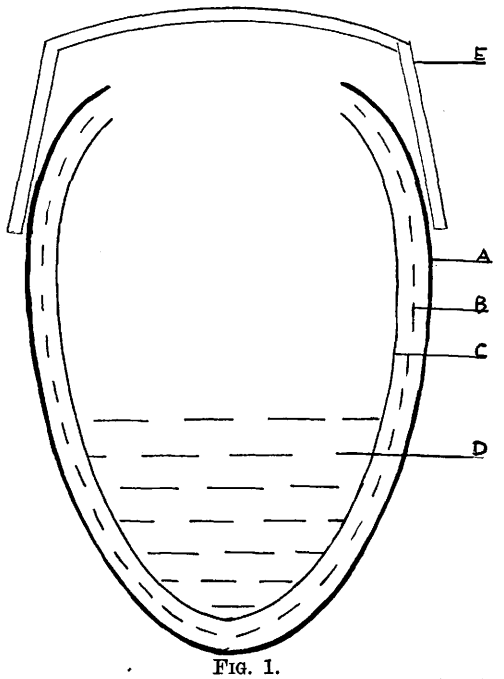

FIG. 1.
A. Egg shell. B. Shell membrane. C. Chorio-allantoic membrane. D. Tyrode's solution. E. Rubber cap.

4. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

were always performed first. After 48 hours' incubation at 37°C all fluids were tested for hemagglutinins. Table I shows the results of several experiments in which the 50% end-points of infectivity obtained with the two methods were calculated by the method of Reed and Muench.(4) It is seen that the titers of allantoic fluid as measured by allantoic sac inoculation were always lower than those obtained with the prepared membrane alone. Since a much larger amount of fluid is introduced into the "empty" eggs, a correspondingly higher amount of virus is also inoculated. This may explain the greater sensitivity observed. Other explanations seem possible.

Discussion. The method may have various applications. It is possible to introduce larger amounts of fluid into the egg than by other methods and this may be of advantage when virus isolation from throat washings are undertaken. The influence of fluids of different composition on virus multiplication and the effect of inhibitors and accelerators of growth can easily be studied by this method which allows virus growth in a practically intact tissue. Moreover, the rate and character of growth of virus in the membrane may be more accurately studied. Such experiments are in progress. The method may have certain advantages for the production of vaccines as virus multiplication can be obtained in a medium of chosen composition, and the presence of urates in the virus fluid is excluded.

Summary. A method is described in which the chorioallantoic membrane of the egg *in situ* is used for the cultivation of influenza virus. The sensitivity of the method is demonstrated and various possibilities for its application are discussed.

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Effect of Proteinuria on Toxicity of Mercurial Diuretics in the Rat. (17542)

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During the past 30 years, organic mercurial compounds have been used widely for the purpose of inducing diuresis. Because of their apparently low clinical toxicity, mercurial diuretics have been used with impunity in the presence of cardiac failure, and, even in the presence of impaired renal function, few clinicians have hesitated to administer them. Studies in animals(1-4) gave results which, while in some degree conflicting, nevertheless indicated that the agents could produce transient or permanent renal damage in doses which were comparable to those recommended for use in man. When clinical application of the mercurial diuretics was begun, it soon became evident that, although there was some indication of renal irritation,(4-6) the clinical toxicity was negligible,(7,8) at least in the absence of severe,

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1. Johnstone, B., and Keith, H. M., *Arch. Intern. Med.*, 1928, v42, 189.

2. Keith, H. M., and Johnstone, B., *Arch. Intern. Med.*, 1929, v44, 438.

3. Möller, K. O., *Arch. f. exp. Path. u. Pharmakol.*, 1930, v148, 56, 67, and 81.

4. Brown, C. L., and Englebach, F., *Medical Papers Dedicated to Henry A. Christian*, Feb., 1936.

5. Barber, M. H., and O'Hare, J. P., *J. Am. Med. Assn.*, 1928, v91, 2060.

6. Sprague, H. B., and Graybiel, A., *New Eng. J. Med.*, 1931, v204, 154.

7. Tarr, L., and Jacobson, S., *Arch. Intern. Med.*, 1932, v50, 158.

8. Berglund, H., and Sundh, E., *Acta Med. Scand.*, 1935, v86, 216.