rather on osmosensitive elements, since they can be evoked by hypertonic glucose and hypertonic sucrose. Whatever influence they may have on the activation of the supraoptico-hypophysial system is a question for further study.

Conclusion. Intracarotid injections of hypertonic saline and sugar solutions in the dog induce characteristic synchronous EEG changes in the olfactory bulb which are independent of influences from the olfactory epithelium or the rest of brain. In the intact brain, these osmosensitive elements in the bulb transmit their nervous impulses caudally along olfactory pathways, but their influence on the supraoptico-neurohypophysial system is unknown.

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Received April 17, 1959. P.S.E.B.M., 1959, v101.

Infectivity of Ribonucleic Acid (RNA) from Type I Poliovirus in Embryonated Egg.* (25005)

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Multiplication of MEFI strain of Type II poliovirus in the chick embryo after prolonged propagation in the suckling hamster is now well established(1,2,3,4). No reports of confirmation of reproduction of Type I or III poliovirus in chick embryo have appeared (see (5)). However, it has been reported(6) that *in vitro* cell cultures from chorio-allantoic membrane of chick embryo can support 10 to 20 transfers of all 3 types of poliovirus, to an extent indicative of multiplication. The present report describes synthesis of Type I poliovirus in embryonated eggs after inoculation by amniotic route with ribonucleic acid (RNA) derived from Type I (Mahoney) poliovirus. Polio-RNA previously exposed to ribonuclease (RNAase) failed to yield the plaque-forming agent.

Materials and methods. Materials and procedures leading to this study have been published(8); additional or modified technics will be described. Mahoney Type I poliovirus grown on human amnion cell culture was obtained from Cutter Labs. (Berkeley, Cal.) as 100-fold concentrate designated E_1. Ribonucleic acid (RNA) preparations were made by phenol method of Gierer and Schramm(9), which consists of 3 extractions with phenol, followed by removal of phenol by ether extraction and subsequent removal of the ether by nitrogen. For our preparations, freshly distilled phenol was used; pH of preparation was adjusted finally by addition of a tenth volume of 0.9 M NaCl containing 0.04 M sodium phosphate at pH 7.6. Aliquots were used which, without additional dilution, had been frozen in a CO_2 box at $-70°C$, and thawed once. Occasionally fresh preparations were used. The plaque forming capacity of polio-RNA was usually tested simultaneously with experimental test fluids. Most viral assay tests were carried out in monolayers of a line of human amnion cells(10), although these were supplemented occasionally with small tests using monolayers of HeLa L5, a clonal line used previously(8). When comparative tests were done, no significant dif-

* Aided by grant from National Fn.

ference in sensitivity of cell lines was observed. Monolayers grown in an atmosphere of 5% CO_2 in air at 35°C, usually for 2 days, were washed with Hanks' solution minus Ca, Mg and PO_4 ions ("simple Hanks' solution"). The same solution was used for second washing when inoculum was tested for presence of whole virus. However, a second washing of alkaline, hypertonic salt solution was used when detection of infectious RNA was sought. The alkaline, hypertonic solution consisted of 0.65 M NaCl containing 0.03 M tris (Sigma 7 to 9 buffer grade (hydroxymethyl aminomethane)) at final pH 7.6. The washing solution was removed as completely as possible(11). The monolayers were inoculated with test fluids, followed after half an hour at room temperature with an overlay of agar; and, after further incubation in 5% CO_2 in air, for 2 or 3 days at 35°C, by a second overlay of agar containing neutral red. Three or 4 hours later, and again the following day, plaques were counted. In the Tables plaque-forming units (PFU)/0.1 ml inoculum are reported. Crystalline ribonuclease (RNAase) of bovine origin was obtained from Armour Co., Chicago. *Antisera and globulin.* Pools of serum from rhesus monkeys, before ("normal") and after hyperimmunization by repeated intramuscular injection of monkey spinal cord and medulla infected with Type I or II poliovirus emulsified in adjuvants served as source of globulins. Globulins were thrice precipitated in half-saturated ammonium sulfate solution, alternately dissolved in distilled water, and finally dialyzed against Earle's solution (BSS) (12).

Fertile eggs, usually 7 days of age, were obtained from Shamrock Farms, Milltown, N.J. After arrival in laboratory they were incubated at 36°C. The inoculum from a syringe with 22-gauge needle was placed into the amniotic sac. To insure proper location of needle, some fluid was drawn up. When crystal clear amniotic fluid was seen in neck of syringe, the inoculum was injected. *Results. Choice of route of inoculation.* Since a prime characteristic of polio-RNA(7,8) is its inactivation by low concentrations of RNAase, an enzyme ubiquitous in animal cells, it was essential to find a route of inoculation such that RNA would come into contact with susceptible cells before its destruction by RNAase. To determine relative desirability of various routes, fluids from "normal" embryonated eggs, 8 to 10 days of age, were compared with a physiological (Hanks') solution for their inactivating effect on polio-RNA. In one test, amniotic fluids from 2 embryos (8 and 9 days old) were compared with 2 sets of controls consisting of Hanks' solution at pH 7.4. One part of each fluid, mixed with 9 parts of 1/50 dilution of RNA, stood 5 minutes at room temperature before inoculation of HeLa L5 monolayers. From 5 plates each the average number of plaques formed in presence of amniotic fluids was 148 and 164 compared with control averages of 109 and 162. In another test 3 amniotic fluids from 9-day-old eggs yielded plaque counts of 85, 80+, 100+, compared to controls with 0.15 M NaCl at pH 8.0, which yielded 65 and 90+. Thus no inactivating effect of amniotic fluid on polio-RNA was observed. On the other hand, when a few yolks and allantoic fluids were tested for their effect on plaque-forming capacity of polio-RNA, some reduction in infectivity was observed. Thus, since amniotic fluids gave no indication of an inactivating effect on RNA in a controlled *in vitro* system, the amniotic route was chosen as most favorable for inoculation of RNA.

A test was done to determine whether amniotic fluid was devoid also of DNAase (desoxyribonuclease) activity. We are indebted to Miss Grace Leidy for testing effect of amniotic fluid from a pool of four 9-day-old developing eggs on the transforming principle (TPd_{SM}) in an *H. influenzae* system using Rd cell culture. No DNAase activity was demonstrable in the amniotic fluid pool under conditions which would detect as little as 1 μg/ml of the enzyme. Thus the amniotic route of inoculation may prove valuable for study of both types of nucleic acid, DNA (desoxyribonucleic acid) as well as RNA, from viruses to which the chick embryo is not susceptible in the usual sense.

Effect of fluids from embryonated egg on whole poliovirus. Chick embryo suspension

and fluids from embryonated eggs were tested for their effect on plaque-forming capacity of E_1 poliovirus (a sample of that from which the polio-RNA had been derived). In 3 tests, there was no inactivating effect on whole Type I poliovirus by 5 to 30 minutes' exposure to 50% chick embryo suspension, 10% yolk, undiluted amniotic or allantoic fluid, as compared with Hanks' solution, under conditions known to favor neutralization by specific antibody. This result is consistent with the finding in many laboratories that Type I poliovirus may survive for long periods in the embryonated egg, although multiplication has not been confirmed. Thus, the failure to multiply is not attributable to any demonstrable neutralizing effect of tissues or fluids of the egg.

Result of inoculation of embryonated eggs with polio-RNA. Fertile eggs of 7 to 8 days' incubation were inoculated with 0.1 ml "undiluted" polio-RNA by the amniotic route. This dose, as assayed on monolayers, varied in different preparations from 300 to 4000 PFU tested in most cases simultaneously with materials recovered from the egg. Higher assays would be expected under optimal test conditions now known(11). Inoculated eggs were replaced in the incubator and at intervals from ½ hour to several days, a few eggs were removed for samples of amniotic fluids and tissues which were frozen once before assay. Each embryo, and each pair of allantoic and amniotic sacs, was ground in glass homogenizer with enough Hanks' solution to make a 50% suspension. 0.1 ml of each suspension or undiluted fluid was tested for plaque-forming capacity on a HeLa L5 monolayer. Results of 7 experiments are summarized in Table I. After incubation for ½ to 2 hours, no plaque-forming agent was recovered from embryos, sacs or amniotic fluids. At 4 hours, about half of the eggs, and at 6-8 hours nearly all eggs yielded plaque-forming agent. It was demonstrated earlier in significant amounts in the embryo and amniotic sac than in amniotic fluid, although the number of plaques observed varied considerably. After 20-24 hours' incubation plaque-forming agent was recovered from a minority of tissues and fluids, and thereafter, at 2 to 7 days, little or none.

Assays in which the alimentary tract with viscera was compared with remainder of embryo failed to yield any significant difference.

Emergence of a considerable amount of the plaque-forming agent earlier and in a higher proportion in tissues (embryo and amniotic sac) than in amniotic fluid may reflect intracellular formation of plaque-forming agent with subsequent release into the amniotic fluid. The time of virus demonstration, with maximum positive results at 6-8 hours, is compatible with growth and release of poliovirus observed in single cells by Lwoff *et al.*(13).

Our earliest demonstration of plaque-forming agent, *viz.*, 4 hours, may indicate a more rapid synthesis of virus from RNA than from intact virus, as compared to Lwoff's experiment. However, further investigation under comparable conditions is necessary to establish this comparison. Thus inability of Type I poliovirus to propagate in the developing chick as observed by several investigators may be due not to inability of cells to synthesize whole virus, when the active nucleic acid gains intracellular position, but rather to failure of absorption or entrance.

Nature of incitant to virus synthesis. To test the possibility that the plaque-forming agent produced in embryonated eggs after inoculation of polio-RNA might conceivably represent survival in the RNA preparation of a trace of residual intact virus present, RNA was exposed to RNAase (final concentration 15 μg/ml) before inoculation of eggs by the amniotic route. This experiment was controlled by an equal number of eggs which received RNA alone. RNA (with or without RNAase, added in dry form) stood 5 minutes at room temperature before inoculation into each of 2 groups of 6 eggs. Amount of "undiluted" RNA inoculated into each egg represented 900 PFU. At 10 minute intervals, from 30 to 80 min, an egg inoculated with polio-RNA alone was opened. The embryo, allantoic and amniotic sacs, and amniotic fluid were removed. No plaque-forming agent was demonstrable from 6 embryos and sacs or 5 amniotic fluids tested by inoculation onto human amnion cell monolayers washed with

TABLE I. Infectivity of Tissues and Fluids Recovered from Embryonated Eggs at Various Intervals after Inoculation of Polio-RNA by the Amniotic Route.

Eggs 7-8 days old at inoc. / Incubation period	Material from inoc. egg / Suspension (50%) or fluid (undil.)	Test No.† I	II	III‡	IV	V‡	VI	VII	Summary Total No. positive / Total No. tested: Emb. and/or sacs	Amniotic fluid
		No. of plaques on each plate								
30-80 min.	Emb. and/or sacs	0,0,0,0,0,0							0/6	
	Amniotic fluid	0,0,0,0,0								0/5
2 hr	*Idem**			0,0,0,0	0,0,0				0/7	
				0,0,0,0	0,0,0					0/7
4 "	"			0,0	12,0,2	7,Cf,0,10,0,45			6/11	
				0,0	0,0,0	0,0,3				1/8
6 "	"		Cf,1,Cf		27,81,36	0,64,Cf,44,35,17			11/12	
			83,0,5		1,3,1	0,0,0,5,0,0				6/12
8 "	"	191,Cf,110,148,177,82			3,0,0,0	Cf,52,Cf,57,23			12/15	
					1,0,0,0	Cf,3,Cf,0,0				4/9
18-24 hr	"		2,0,0			4,0,0,0,0,0	156,53	0,0,0,0,0,0,0,0	4/20	
			7,1,0	8,0,16,12,75,0,0,0		0,0,0,0,0,0	89,Cf	0,0,0,0,0,0,0,0		8/28
2 days	"		0,0,0						0/3	
			23,0,0							1/3
5-7 days	"		0,0,0				1,0		1/5	
			0,0,0				3			1/4

Test procedure: 0.1 ml each fluid was inoculated onto monolayer of human amnion cells.

* As in the first (30-80 min. incubation) group, the top line indicates Emb. and/or sacs, and the bottom line Amniotic fluid throughout.

† Plaque-forming capacity of materials in each test was assayed simultaneously, after single cycle of freezing and thawing followed by centrifugation.

‡ In tests III and V, all test fluids were exposed to RNAase (at final conc. of 10 μg/ml) for 5 min. at room temperature before inoculation. Thus plaque formation was interpreted as caused by intact virus and not by infectious RNA. RNA preparations to inoculate eggs proved infectious when tested on 5 occasions simultaneously with materials removed from eggs.

alkaline, hypertonic salt solution. However, after 8 hours, all embryos and sacs of 6 eggs which received RNA alone yielded plaque-forming agent with counts/plate of 191, 250, 110, 148, 177 and 82. In contrast, comparable materials recovered from 6 eggs inoculated with RNA, and exposed to RNAase immediately before inoculation, yielded, after a similar 8-hour period, no plaque-forming agent. The outcome of this experiment makes it highly improbable that plaque-forming agent recovered from the egg originated from a trace of active virus remaining in the nucleic acid preparation. On the contrary, the experiment demonstrated that the inciting agent proved susceptible to action of RNAase, a cardinal property of the infectious nucleic acid.

Identification of plaque-forming agent formed in embryonated egg. The effect of RNAase and of anti-I globulin on plaque-forming capacity of a pool of embryos and sacs from eggs harvested 8 hours after inoculation with RNA alone was measured. The plaque-forming agent recovered from eggs yielded in one test essentially the same number of plaques after exposure for ½ hour at room temperature to RNAase (169 and 125) as to BSS (141, 182, and 119) but none after exposure to anti-I globulin, thus fulfilling requirements for identification as Type I poliovirus. The result was confirmed in 3 other tests. The type-specificity of the plaque forming agent, enhanced in titer by passage through human amnion cell culture, was determined. The freshly harvested virus was titrated in presence of 1/10 dilutions of monkey sera. In presence of "normal" serum, and of anti-II serum, 7.3×10^7 and 5.5×10^7 PFU/ml, respectively, whereas in anti-I serum, 5.3×10^2 PFU/ml were observed, thus demonstrating type-specificity of poliovirus recovered from eggs, after human amnion cell passage.

Egg passage of plaque-forming agent. To determine whether Type I poliovirus synthesized in eggs in response to inoculation with polio-RNA might multiply on passage through embryonated eggs, Type I virus recovered 8 hours after inoculation of eggs with polio-RNA was passed by the amniotic route. A suspension of embryos and sacs, containing 160 plaque-forming units/0.1 ml, was inoculated into eggs. Embryos and sacs harvested 8 hours later yielded almost no virus, while a pool of allantoic and amniotic fluids yielded an average of 30 plaques/0.1 ml, suggesting survival of some of the inoculated virus. In 2 experiments, eggs harvested at 18-24 hours yielded no plaques from tissues or fluids.

The possibility that larger doses of poliovirus recovered from eggs inoculated with polio-RNA might be more effective in passage led to attempts with virus enhanced in titer by passage through human amnion cell culture. Such virus was inoculated into eggs in doses from $10^{4.8}$ to $10^{6.8}$ PFU. After 6 days(4), 1/100 to 1/1000 of amount inoculated was recoverable. On second passage, only a few plaque-forming units were demonstrable, and none on third passage. Virus survival in the egg was comparable to that of Type I E_1 poliovirus (passaged in parallel) which had had no previous contact with the egg. Thus there was no indication that the egg-derived virus could multiply in the embryonated egg. Whether virus recovered from eggs could be adapted on egg passage has not been determined. Virus recovered from eggs has been kept in frozen state for future studies.

Summary. 1) Phenol extraction of Type I poliovirus yielded a plaque-forming agent which is presumably ribonucleic acid (RNA) because it is inactivated by RNAase. Such ribonucleic acid derived from Type I (Mahoney) poliovirus induces formation of plaque-forming agent in the embryonated egg, inoculated by amniotic route. The plaque-forming agent recovered from embryo tissue, sacs, and fluids has been identified as type specific, Type I poliovirus. Highest yields occurred at 6 to 8 hours after inoculation of eggs with polio-RNA. 2) Polio-RNA exposed to RNAase immediately before inoculation into embryonated eggs failed to incite formation of plaque-forming agent. This establishes that it is RNA itself, and not some undetectable trace of intact virus, which incites production of whole virus in the egg. 3) Propagation of virus recovered from eggs by further

passage in embryonated egg have not succeeded. 4) This demonstration of production of Type I poliovirus from nucleic acid in the embryonated egg, without subsequent multiplication, provides a system for biochemical study of genesis of whole virus from its nucleic acid. An advantage of the system is that the inciting agent (infectious RNA) can be differentiated completely, by its susceptibility to the action of RNAase, from whole virus progeny.

We acknowledge the capable technical assistance of Olga Van Damme and Susan Koenig.

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Received April 20, 1959. P.S.E.B.M., 1959, v101.

Antagonism of Succinylcholine to Chlorpropamide Induced Hypoglycemia. (25006)

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Hypoglycemic activity of 1 (p-chlorobenzenesulfonyl) 3-N propylurea (chlorpropamide, Diabinese®) has been well established experimentally(1) and clinically(2). Following high doses of chlorpropamide in animals, a decrease of spontaneous motor activity and drowsiness were noted. This prompted an electroencephalographic study in the course of which cats were immobilized with succinylcholine. Surprisingly enough, severe hyperglycemia was found and normally effective doses of chlorpropamide did not lower blood glucose. This study was designed to elucidate this antagonistic action.

Methods. Forty-five cats and 100 rats were employed. Cats of both sexes ranging in weight from 2.5 to 3.5 kg were fasted 16-18 hr. Anesthesia was induced by intraperitoneal injection of pentobarbital sodium 40 mg/kg. A tracheal cannula was inserted and femoral vein was cannulated for injection of various agents and collection of blood samples. Whenever necessary, artificial respiration was maintained by Palmer Pump using 20 to 30 cc of air. Bilateral adrenalectomy was performed by lumbodorsal approach. Such animals were maintained 24 hr by subcutaneous injection of 1 mg/kg of cortisone. Male albino Wistar rats weighing 275-350 g were used necessitating artificial respiration. A tracheal cannula was inserted and attached to a Harvard Pump. For all other experiments, rats weighing 140-175 g were used. Bilateral adrenalectomy was performed 2 days prior to testing; animals were maintained by giving 1% saline in drinking water. Partial hepatectomy was performed under ether anesthesia 1 day prior to testing with removal of median and left lobes which comprise about 70% of liver tissue(3). All rats were fasted 18 hrs. The sodium salt of chlorpropamide was administered intravenously. Other agents were given as specified. Blood glucose determinations were performed with Auto Ana-