the acapnia resulting from the increased respiratory volume occurring under oxygen deficiency. If, however, artificial respiration is instituted, the CO_2 tension in the blood remains constant; and any changes in the efficiency of CO_2 must be attributed to its effect on the circulatory rather than the respiratory system. Under these conditions similar results were obtained. Here again the rise in blood pressure was greater in the oxygen deficient gas mixture containing 4%CO_2 than corresponded to the sum of the rises obtained when either one of the two factors was administered separately. This potentiation of the CO_2 and oxygen deficiency effect on the blood pressure must be considered to be of utmost value in maintaining an adequate circulation through the brain. The results indicate that the beneficial effect of CO_2 in offsetting oxygen deficiency is due not only to its influence on respiration but to augmenting greatly the rise in blood pressure which occurs under the conditions of oxygen deficiency.

Summary. The rise of blood pressure caused by oxygen deficiency is greatly augmented or potentiated by small amounts of CO_2 which in themselves have no effect upon blood pressure. This potentiation is present not only in the animal capable of respiratory adjustments, but also when the minute volume of ventilation is kept constant by means of artificial respiration. These conditions indicate that the circulatory system, *per se,* does play a major part in the adjustment to oxygen deficiency brought about by small amounts of CO_2.

9158

Multiplication of Yellow Fever Virus in the Developing Chick Embryo.

JOHN E. ELMENDORF, JR., AND HUGH H. SMITH. (Introduced by J. H. Bauer.)

From the Laboratories of the International Health Division, Rockefeller Foundation, New York.

Since Woodruff and Goodpasture[1] first discovered that fowl pox virus could be grown on the chorio-allantoic membranes of developing chick embryo in the egg, many workers have reported success in the propagation of other viruses by this method. It was shown[2, 3]

[1] Woodruff, A. M., and Goodpasture, E. W., *Am. J. Path.*, 1931, **7**, 209.

[2] Haagen, E., and Theiler, M., *Zentralbl. Bakter., Abt. I, Orig.*, 1932, **125**, 145.

[3] Lloyd, W., Theiler, M., and Ricci, N. I., *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1936, **29**, 481.

that yellow fever virus could be grown *in vitro* in a medium containing chick embryonic tissues. It was considered of interest to determine whether this virus could be maintained in the living embryos. Using the window-technique of Goodpasture, the first attempts were made with a strain of virus long cultivated *in vitro* in a medium consisting of monkey serum-Tyrode solution and minced chick embryo. After the culture flasks were incubated for 3 to 4 days at 37°C., the supernatant fluid was pipetted off and a drop deposited on the chorio-allantoic membrane of several eggs after a window-flap had been cut in the shell. At the end of a 4-day incubation period at 37°C., virus was demonstrable in considerable quantities by the intracerebral test in mice in both the embryonic fluid and in the embryo itself. It was found possible to passage this virus from egg to egg using the fluid, the membranes, or a suspension of embryonic tissue as the inoculum.

In order to simplify the technique of egg passage, a method of direct inoculation of the embryo itself was tried and was found to be satisfactory. Eggs having living embryos, preferably 7 days old, were selected. After cleansing the shell with 95% alcohol, a small hole was drilled with a sharp dissecting needle directly over the embryo as seen by the transmitted light. Care was taken not to pierce deeper than was necessary to puncture the outer membrane. Using a tuberculin syringe with a 1-inch 27-gauge needle, the embryo was stabbed by a quick thrust of the needle and 0.03 cc. of the virus-containing material was injected. After the needle was withdrawn, the small hole in the shell was sealed with liquid paraffin. The eggs were then incubated at 37°C. for the desired length of time and were turned every day in the customary manner.

After 4 or 5 days' incubation, the eggs were again examined by a transmitted light. Those containing living embryos were selected as a source of virus for further passages. After washing with 95% alcohol, the egg was placed in an egg cup with the air sac up. The shell was cut away with sterile scissors, the outer membrane ruptured, and the embryo lifted out by the neck. Usually 2 or 3 embryos were pooled for each passage. They were weighed, placed in a sterile mortar, chopped up with scissors, and then ground up thoroughly with sand. A 50% suspension was made of this triturated material in normal saline solution. The suspension was centrifuged for about 10 minutes at low speed to throw down the larger tissue fragments and the supernatant fluid used to inoculate a new series of eggs. A test for the presence of virus was made at each passage by inoculating a group of 6 mice intracerebrally with the supernatant fluid. At frequent intervals passage material was frozen and desiccated for preservation.

Strain of virus adapted to chick embryo tissues. The strain of yellow fever virus which was successfully maintained in developing chick embryo was one which had already been greatly modified in both its neurotropic and viscerotropic properties by prolonged cultivation *in vitro* in a medium consisting of chick embryo tissues in serum-Tyrode solution. Sixty consecutive egg-to-egg passages have been made with this virus. Successful transfers were made at intervals as short as 2 and as long as 8 days after inoculation. The optimum age of embryos for this purpose was found to be 7 or 8 days. When younger embryos were used, many deaths in the first 2 days after inoculation were encountered.

There is definite evidence to indicate that multiplication of the virus takes place in the embryo. The titer of the strain in the tissue cultures used to initiate this series was low, varying at different subcultures from 1 in 250 to 1 in 2000. The titer of the virus in the embryo tissue, on the other hand, was found to be 1 in 17,000 after 6 egg-to-egg passages, and 1 in 195,000 after 12 passages.

Strain of virus adapted to mouse embryo tissues. Another strain of yellow fever virus used in these experiments was one which had been cultivated continuously for a period of nearly 3 years in a medium consisting of mouse embryo tissue and 10% normal monkey serum in Tyrode solution. Developing chick embryos were inoculated with this strain in the manner described above and a multiplication of the virus in the embryos was readily demonstrated. Repeated titrations indicated that the virus concentration of this strain in tissue cultures varied from 1 in 5000 to 1 in 20,000. Infected chick embryos, on the other hand, proved to be infectious after 4 days of incubation in dilutions of 1 in 48,000 to 1 in 100,000, indicating a much higher concentration of virus than was observed in the tissue cultures.

Unmodified strains of yellow fever virus. Using the technique described above, 4 additional strains of yellow fever virus were successfully cultivated in developing chick embryo. Two of these strains, the Asibi and the French, were originally isolated in Africa and are highly pathogenic for laboratory animals. The other two, E-5 and AFB, were obtained from Brazil and possess only a mild degree of virulence. These unmodified strains were not so readily adapted to the developing chick embryo as were the tissue culture ones. Only one of them, the E-5, was successfully established at the first attempt, and subsequent transfers from egg to egg were continued without difficulty. With the remaining 3 strains, several attempts failed before they were successfully established and subsequent transfers to fresh embryos gave positive results. After a

strain had been successfully carried through the first 2 or 3 passages, no difficulty usually was encountered in continuing its propagation in this manner. Transfers were made at 4- to 5-day intervals, at which time the virus had reached its highest concentration, although it was demonstrated in considerable quantities in embryos as late as 11 days after inoculation.

These strains of yellow fever virus have not been carried long enough in developing chick embryo to indicate whether a fundamental change in virulence and tissue affinity of the virus will eventually take place. In one test a Rhesus monkey was inoculated with the French strain of virus that had been carried through 10 egg-to-egg passages. This animal died promptly of typical yellow fever, indicating that no modification in the virulence of the virus had taken place as a result of serial passage through 10 developing chick embryos. In another test, a strain of virus, which had been carried through 165 subcultures in chick embryo tissue—normal monkey serum—Tyrode solution medium, and in addition passed through 18 serial direct transfers from one developing chick embryo to another, was tested in 4 normal Rhesus monkeys. These animals were inoculated intracerebrally with 1 cc. amounts of 1 in 5, 1 in 50, 1 in 500, and 1 in 5000 dilutions. Three of the 4 monkeys showed fever after an incubation period of 10 days. The one which had no fever showed signs of encephalitis on the 7th, 8th, and 9th days. Four weeks after the inoculation, their sera were tested for protective properties against yellow fever virus, and the animals were also given a test dose of highly virulent Asibi virus to determine their immunity. All 4 monkeys showed immune substances in their serum and all survived the test dose, indicating that they had become immunized as a result of the inoculation with chick embryo passage virus.

Conclusions. 1. Yellow fever virus, unmodified strains as well as those adapted to tissue cultures *in vitro,* can be successfully cultivated in developing chick embryo. 2. Direct transfers can be maintained by the use of the chorio-allantoic membrane, embryonic fluid, or a suspension of minced tissues of the embryo. 3. A method of direct inoculation of the embryo in the egg with a tuberculin syringe and fine needle has been found satisfactory for routine use. 4. The virus strains studied showed no evidence of modification in the relatively few passages so far made.