influenza B in 1965 in a controlled field study. Sera from normal adults collected in June of 1965 often showed low antibody titers and this was consistent with the sporadic occurrence of influenza B which was recorded in the U.S.A. during the 1964-65 respiratory disease season. The findings in the study may be of value as a guideline for judging the significance of future antigenic deviation of influenza B viruses. The 1965 influenza B viruses were unique in being sensitive to a nonspecific inhibitor in chicken and human sera which was "created" by treatment with periodate. This finding emphasized the importance of establishing the utility, with each virus, of the methods used to remove nonspecific inhibitors prior to their routine application.

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Persistent Polioviral Infection of the Intact Amniotic Membrane. II. Existence of a Mechanical Barrier to Viral Infection. (31648)

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In the animal host dissemination of virus within a given tissue is often limited, and diffuse cellular destruction is seldom observed. The liberation of interferon(1) and other antiviral substances by infected cells may account in part for some instances of viral inhibition, although it seems unlikely that such mechanisms are solely responsible for the localization of viral lesions. To assess the susceptibility of animal cells to a given virus *in vivo* one must consider the possibility that viral particles may not always have free access to these cells. Thus the arrangement of the stroma or a layer of mucus covering the cells may prevent viral adsorption to potentially susceptible cells.

In contrast to this *in vivo* cellular resistance, cells cultivated under artificial conditions *in vitro* are usually susceptible to infection by numerous viruses. Poliovirus type I for example multiplies readily in human amnion monolayer cell cultures inducing complete cellular destruction(2). If, however, the natural arrangement of cells of the membrane is left undisturbed and the membrane maintained in a nutritive medium, the behaviour of poliovirus in this system is entirely different from that observed in monolayer cell

cultures. Thus, in the polioviral infected amniotic membrane virus is liberated for several months, extensive cellular destruction does not occur and only a small proportion of the cells is found to be infected at a given time(3). The difference between these two systems, the intact membrane and the monolayer culture stems from a difference in the facility with which poliovirus adsorbs to and penetrates the cells(3). Even when the membrane is maintained *in vitro* for 3 weeks, and then exposed to large amounts of poliovirus, less than 1% of the cells are found to be infective. This finding is in contrast to monolayer cell cultures where the whole cell population can readily be infected by poliovirus and destroyed in a single cycle of multiplication.

The results reported here suggest that in the histologically intact membrane (as perhaps in certain tissues *in vivo*) and intercellular matrix may act as a barrier to viral infection limiting progressive infection of contiguous cells. After trypsinization of the aminos, cells at first retain this intercellular substance since it is apparently not destroyed by this proteolytic enzyme. As the cells spread on the glass surface, this substance diminishes and concomitantly the amnion cells become susceptible to infection by poliovirus.

Materials and methods. Human amniotic cells. The methods of obtaining human amniotic membranes, their maintenance *in vitro* and the cultivation of amnion cells has previously been described(3).

Virus, poliovirus type I, LSC-2ab (Sabin) was propagated in BSC cells (obtained from Microbiological Assoc., Bethesda, Md.) and titrated either by tube dilution techniques or by conventional plaque assays as previously described(4).

Titration of poliovirus type I in human amnion cells. Trypsinized amnion cells were distributed in Kahn tubes. Twenty-four hours later the medium containing cells non-adherent to the glass surface was removed and fresh medium added. Poliovirus type I (final concentration 10^4 TCID 50/ml) was added simultaneously to these cultures and to amnion cells cultivated as a monolayer for 3 weeks. Each day thereafter 8 cultures from each group were frozen and maintained at —20°C until testing. Prior to testing, cultures were frozen and thawed 3 times in order to release intracellular virus. Titers of poliovirus therefore represent the sum of intra- and extracellular virus.

Methods of staining amniotic membrane. Membrane strips (5 × 5 cm) were pinned on a cork block (open in the center). After washing with distilled water, the membrane was exposed to a 0.2% solution of silver nitrate under a strong light source. After 2 minutes exposure, the membrane was thoroughly washed and fixed in 90% alcohol. The tissue was then dehydrated and placed on a glass slide and a coverslip was added with permount.

Phagocytosis of carbon particles. Cultured amnion cells or membrane cells were exposed to a suspension of carbon particles (Pelikan, Günther Wagner, particle size: 200-300 Å) for periods varying between 30 minutes to 5 days. The cells or tissues were thoroughly washed with saline solution, fixed and stained with hematoxylin and eosin.

Results. Response of human amnion cells to poliovirus type I. Demonstration of polioviral multiplication in the aminos was facilitated by elimination of adsorbed neutralizing antibodies from the tissue by repeated changing of the nutritive medium for the first 2-3 days. Regardless of the initial multiplicity of infection, only a small fraction of the cell population yielded infectious virus. As shown in Fig. 1, virus was liberated continuously for days. Likewise after trypsinization and cultivation on a glass surface, amnion cells were still found to be relatively insusceptible to polioviral infection as previously reported by Holland(5). The difference in response to poliovirus I of cells cultivated for several weeks and freshly trypsinized cells is illustrated in Fig. 2. In the former system viral multiplication occurred without any significant lag period whereas in cultures of freshly trypsinized cells viral multiplication was not observed for 4-5 days. In the 3-week-old monolayer cultures the sharp increment in virus yield suggested that the vast majority of the cells participated in the synthesis of poliovirions. In cultures of freshly trypsin-

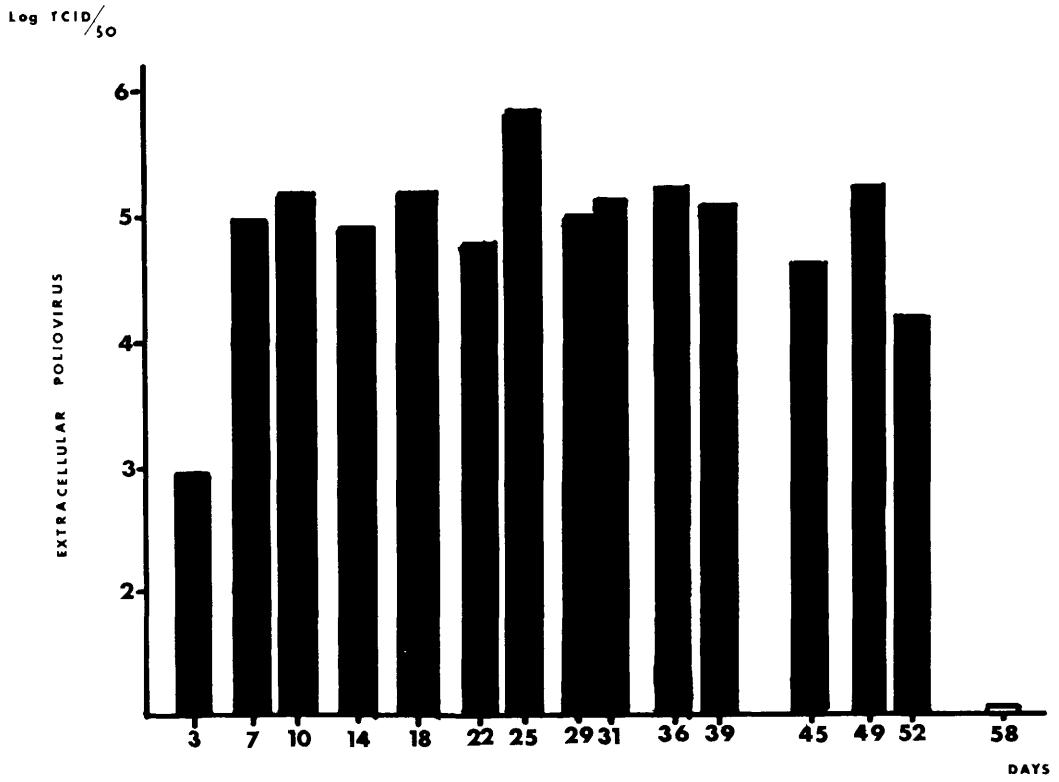

FIG. 1. Multiplication of poliovirus type I (Sabin) in intact human amniotic membrane.

ized cells, however, the daily increment of viral synthesis was less pronounced. This observation was compatible with the development of susceptibility to infection by poliovirus by increasing numbers of cells.

Demonstration of an argentophil matrix surrounding amnion cells. Since cultivated amnion cells differed in appearance from cells of the intact membrane it seemed of interest to follow the morphologic alterations attendant upon trypsinization and cultivation and to correlate such changes with susceptibility to poliovirus.

Amnion membrane strips treated with a silver nitrate solution revealed the presence of a well demarcated argentophil envelope separating the cells (Fig. 3). This technique of silver impregnation described by Ranvier (6) was first employed to delineate the mesothelium of the peritoneum, since silver grains emphasized the intercellular matrix. Shortly after trypsinization individual amnion cells or clumps of cells adhered to the glass sur-

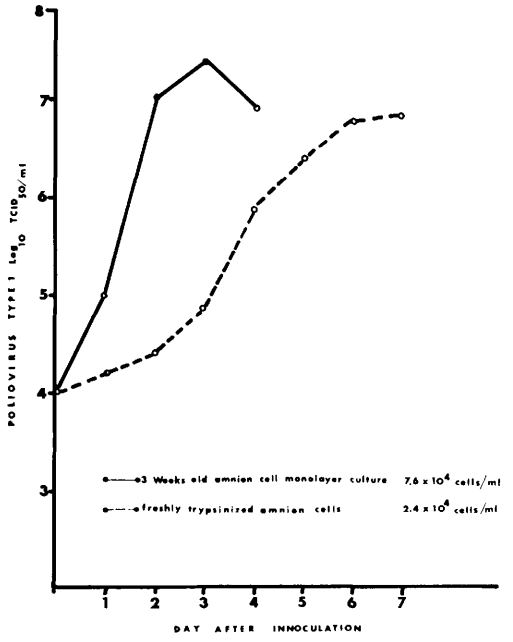

FIG. 2. Response of human amnion cells to poliovirus type I.

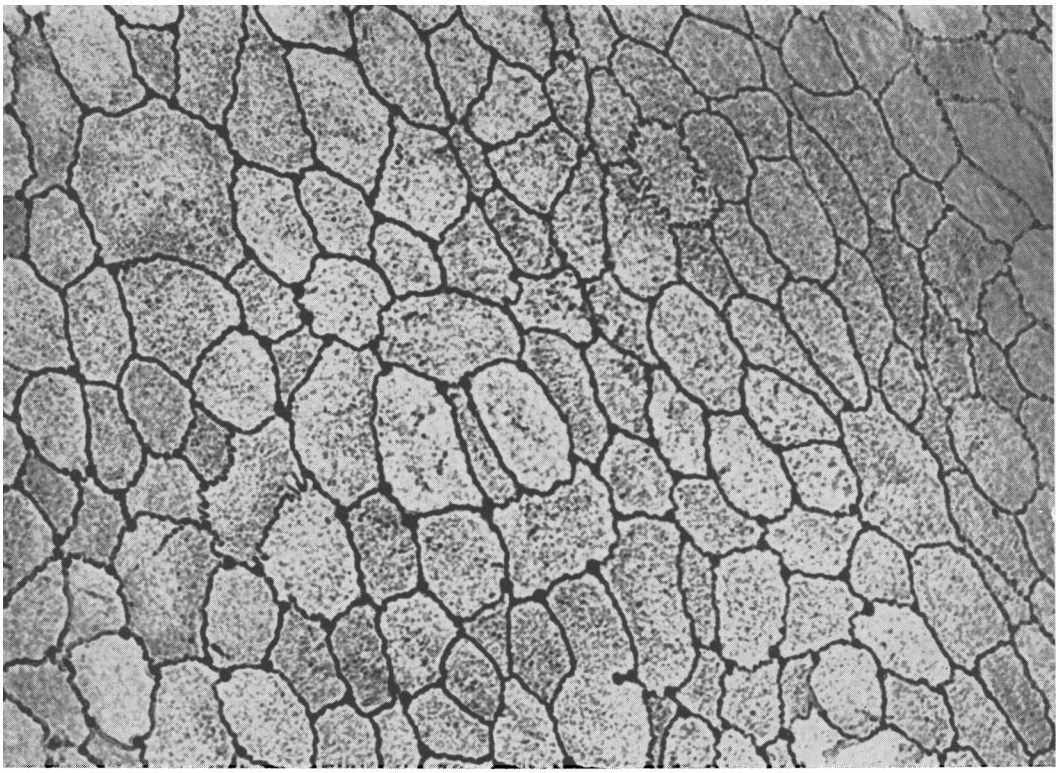

FIG. 3. Human amniotic membrane treated with silver nitrate (800×).

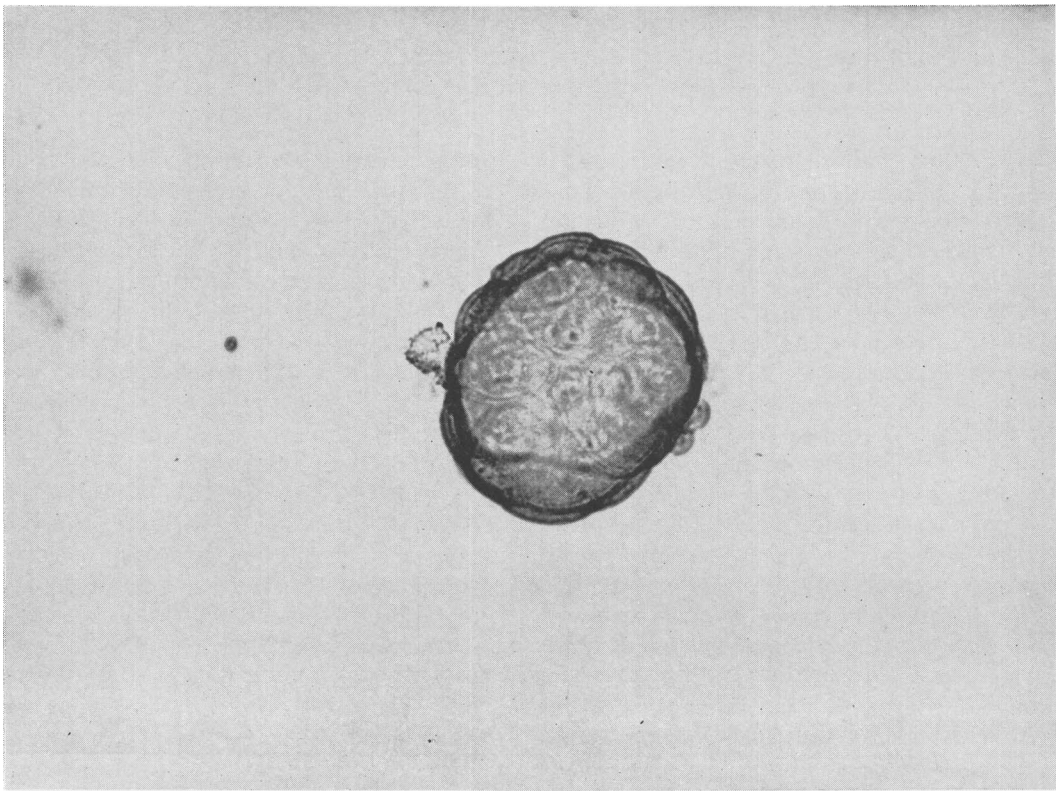

FIG. 4. Human amniotic cells 24 hours in culture—Note thick argentophil envelope (1800 ×).

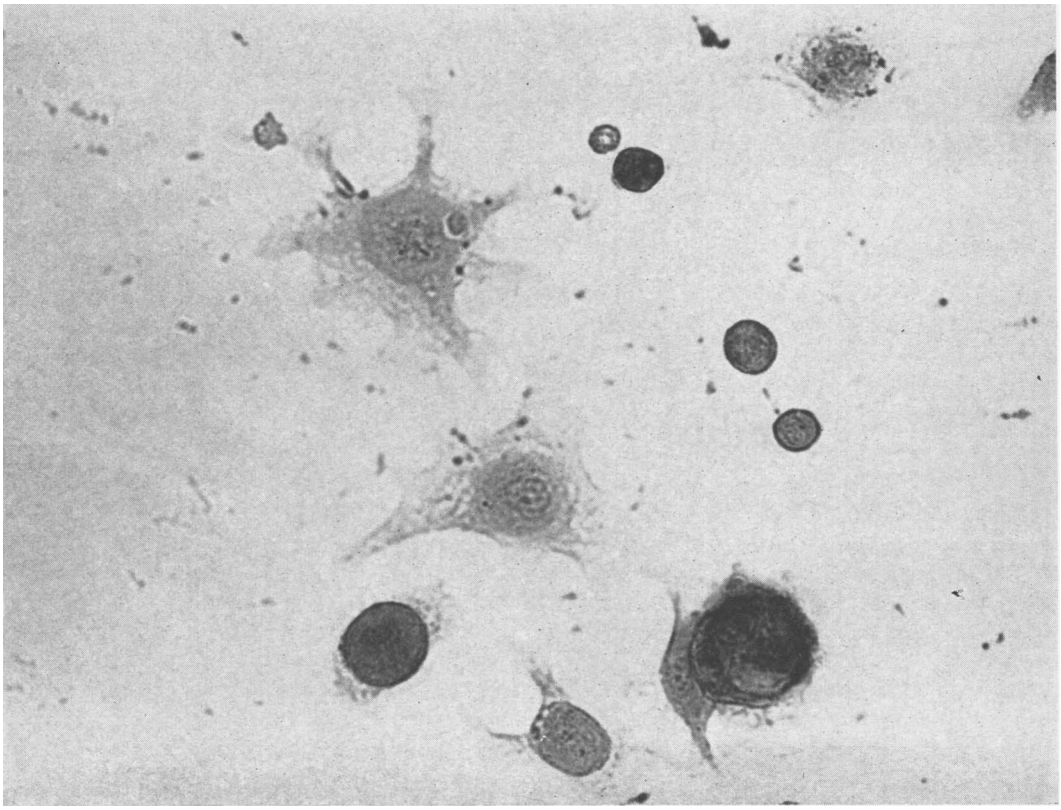

FIG. 5. Human amniotic cells in culture (48 hours). Note argentophil matrix around some cells and the absence of this matrix around other cells as they spread out on the glass surface.

face. When stained with silver nitrate, a thick envelope was easily visualized around either single cells or clumps of cells (Fig. 4, 5). These cells were spherical in configuration and the cytoplasm stained deeply with eosin. In the ensuing days, amnion cells flattened and spread out on the glass. The cell boundaries sharply delineated at first, became less distinct; the cytoplasm stained less intensely with eosin and after impregnation with silver nitrate the intercellular matrix was seen to be considerably diminished (Fig. 6). As can be seen in Fig. 2 polioviral multiplication began at this time in duplicate cultures of these cells.

Incorporation of carbon particles by human amnion cells. Since animal cells may incorporate viral particles by pinocytosis, it was of interest to compare the phagocytic capacity of amnion cells of the membrane and amnion cells in monolayer culture. When the intact membrane was exposed to a suspension of carbon particles, these formed a film above the cellular layer and only a rare amnion cell was observed to contain carbon. Likewise carbon particles adhered to the surface of freshly trypsinized cells but were rarely incorporated by these cells. Cells of monolayer cultures, however, readily engulfed this material (Fig. 7b). When these cultures were exposed to carbon particles for 1 hour, the cells at the periphery of the cell sheet were first to take up the carbon, although after more prolonged exposure almost all the cells incorporated these particles. It is of interest in this regard that the cytopathic effect of several animal viruses in cell culture is also often observed initially at the periphery of the cell monolayer, before affecting the more closely packed cells at the center of the cell sheet. As further proof of the phagocytosis of carbon particles by amnion cells, monolayer cultures, previously exposed to carbon were treated with a mixture of versene-trypsin;

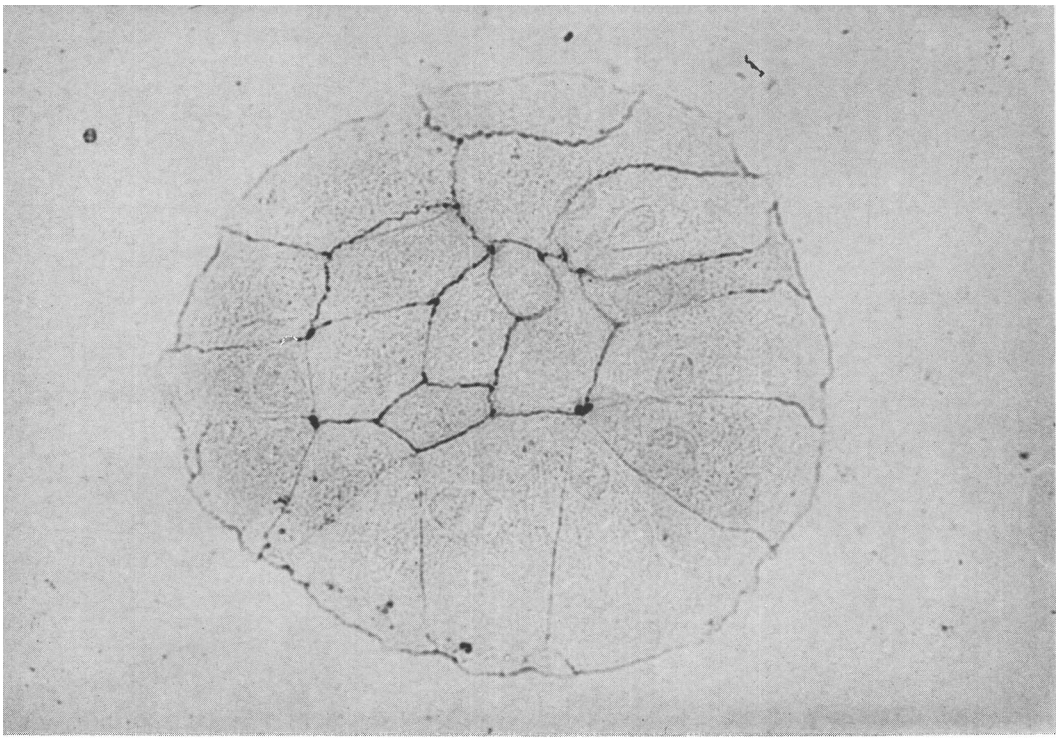

FIG. 6. Human amnion cells 96 hours in culture—Note diminished impregnation by silver (800 ×).

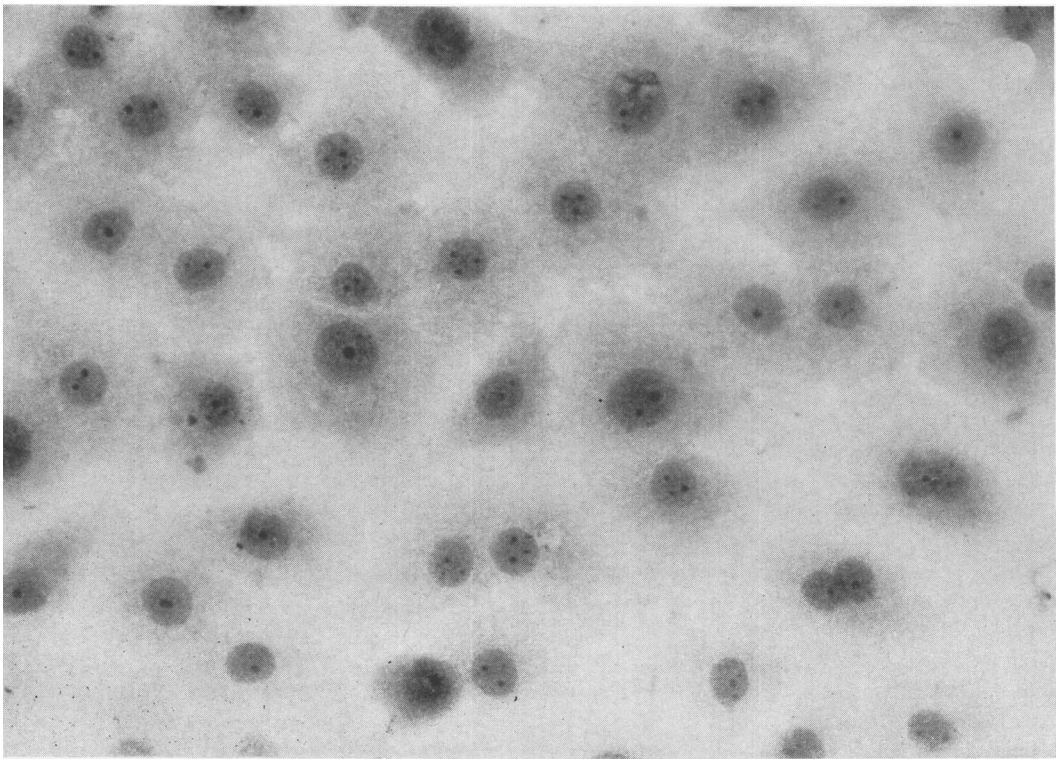

FIG. 7a. Human amnion cells in culture 1 month control (600 ×).

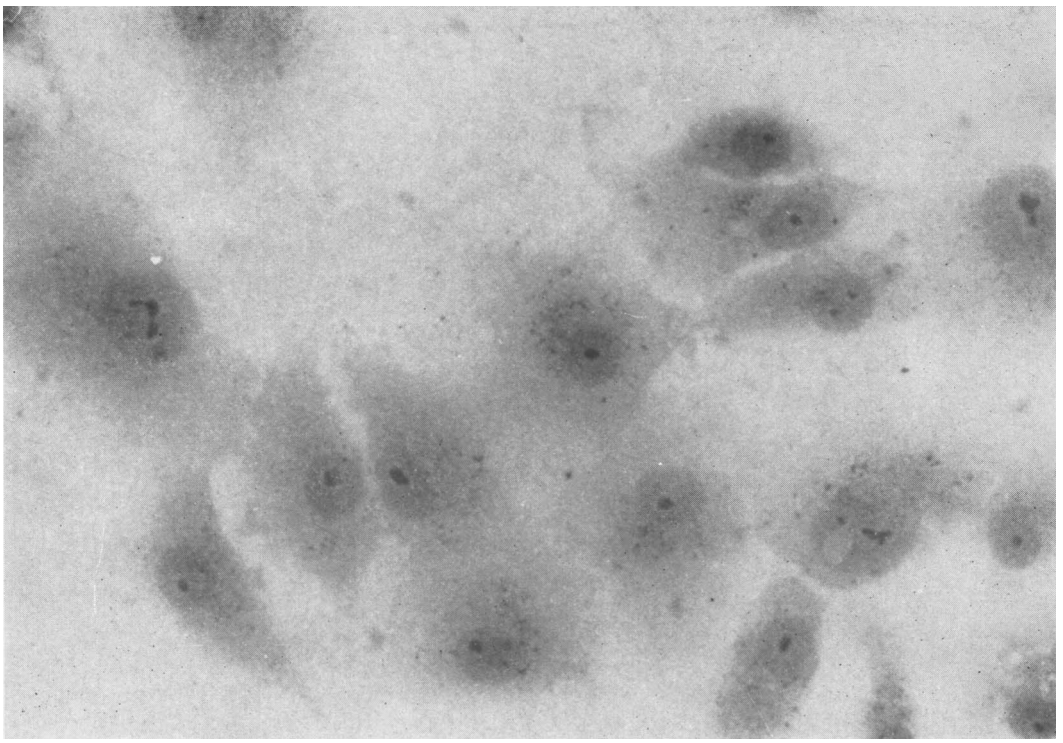

FIG. 7b. Human amnion cells in culture 1 month exposed to carbon particles (600 ×).

the cells sedimented by centrifugation and fixed in Bouin's solution. Histologic sections of the cell block revealed the presence of carbon particles within the cytoplasm of amnion cells.

Liberation of globulins by the amniotic membranes maintained in vitro. As previously described(3) the diffusate of the amniotic membrane maintained for 24 hours in a balanced salt solution neutralized the infectivity of poliovirus. By various methods it was demonstrated that this effect was due to the presence of serum neutralizing antibody which had diffused from the interstices of the thoroughly washed membrane into the nutritive medium. In the course of comparing the protein electrophoretic patterns of this diffusate with the pattern of the corresponding umbilical cord serum, it was apparent that the percentage (of total protein) of β globulin of the diffusate was far greater than the percentage of β globulin found in the serum. Thus in each of 16 samples tested the percentage of β globulin of the diffusate was found to be 1.5-6.8 times that of the percentage of β globulin in the corresponding umbilical cord serum (Fig. 8). As tested in standard neutralization tests, this fraction, however, did not add significantly to the antipoliovirus activity of the diffusate. Thus if this β globulin is derived from the intercellular ground substance, failure of poliovirus to infect the amniotic membrane is not due to a direct antiviral effect at the cellular surface.

Discussion. To explain the susceptibility to poliovirus of amnion cells cultivated *in vitro* as opposed to the resistance to infection of cells of the intact aminos and freshly trypsinized amnion cells, it has been suggested that cultivated cells acquired specific viral "receptors" in the course of cultivation (5,7). These "receptors" which permitted penetration of virus within the cell were presumably not present either on membrane cells or on freshly trypsinized cells. Our results suggest an alternative hypothesis: Cells of the intact membrane are embedded in an

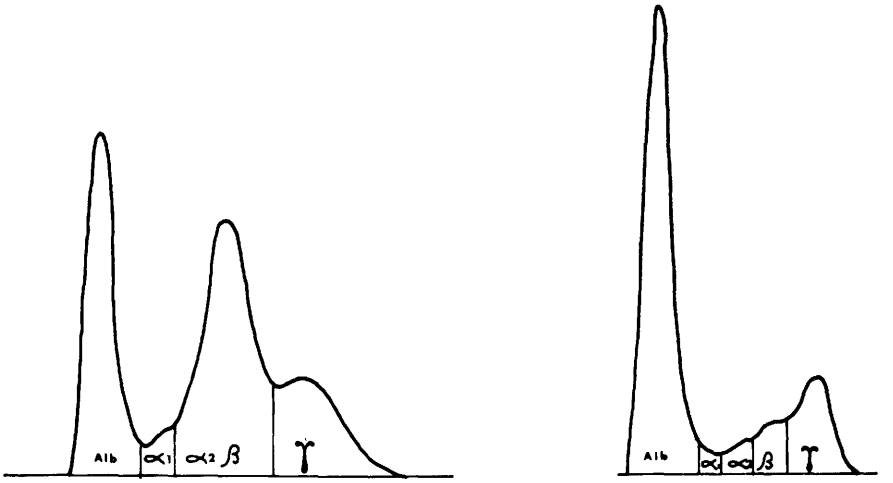

FIG. 8. Electrophoretic pattern of diffusate from amniotic membrane and electrophoretic pattern of serum.

intercellular matrix, which restricts significant cellular phagocytic activity. Thus neither virus nor larger particulate material gains easy access to the cell. When the amnion cells are trypsinized and first adhere to the glass surface this intercellular substance is still present, forming an envelope around the aggregates of cells and also around individual cells. At this stage, neither virus nor particulate matter is taken up by cells in significant amounts. In the ensuing few days, the cells multiply, flatten and spread out on the glass. The intercellular substance is markedly diminished and the cells assume a morphologic and physiologic character they did not previously possess. Edwards and Fogh(8) pointed out that trypsinized cells become "mobile self-contained, free living, unicellular organisms of ameboid form." It is therefore not surprising that at this stage they exhibit phagocytosis of carbon particles and pinocytosis of poliovirions.

These observations may bear on the pathogenesis of polioviral and other enteroviral infections in man. In attempting to define the natural human enteric viral flora, Kalser and his coworkers(9) aspirated by means of a polyvinyl tube the intestinal contents of 37 subjects. Although enteroviruses were consistently recovered in their laboratory from fecal material they were unable to isolate these agents from samples taken at different levels of the jejunum, ileum and colon. One may speculate that the epithelial cells of the intestine, situated closely to each other and covered by a layer of mucoid material, resemble the intact amniotic membrane *in vitro* in their response to enteroviruses. It is probable that if poliovirus or other enteroviruses do multiply in the intestinal lining cells, they do so to a very limited extent.

Evans and his coworkers(10) found that poliovirus multiplied readily in the traumatized subcutaneous tissue of experimentally inoculated monkeys. One may speculate that as a result of injury the natural arrangement of the cells to each other was disturbed, and in the course of tissue repair new cells appeared which were not yet fully integrated into the local structure. These cells may be highly phagocytic and may have been responsible for the viral multiplication observed.

The role of the ground substance of the mesenchyme as a barrier to viral dissemination was emphasized by the work of Duran-Reynals(11,12) and Sprunt(13). Duran-Reynals underlined the importance of viewing viral infection *in vivo* as more than a "conflict between the infectious agent and the cell." His comments seem pertinent to the problem we have investigated: "The concept of the cell is incomplete as long as one does not take into account the substances which bathe it—or of any other intercellular matric"(12).

Summary. Human amnion cells cultivated

in monolayer cultures can easily be infected by poliovirus I, whereas cells of the intact membrane and freshly trypsinized amnion cells are for the most part resistant to infection. Silver impregnation of the histologically intact membrane revealed the presence of a well demarcated intercellular matrix. As the trypsinized amnion cells flatten and spread out on the glass surface, this intercellular substance diminishes considerably, the cells demonstrate marked phagocytic activity and they become susceptible to polioviral infection. It is suggested that this matrix prevents penetration of the virus within the cell either by restricting viral adsorption or by restricting phagocytic activity.

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Barometric Pressure and Seizures.* (31649)

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Several factors have been shown to modify the threshold to seizures in animals and man (1), but the possible importance of changes in barometric pressure in explanation of periodic variations in seizure susceptibility has been investigated infrequently. Petersen(2), in an extensive treatise concerning the effects of the weather on disease, reported mortality and draft statistics which pointed to a relation between meteorological disturbances and the precipitation and severity of epileptic seizures. Furthermore, Tille(3) observed that the incidence of febrile convulsions in young children was greater at times of cold weather fronts than during passage of warm fronts.

In previous investigations of barometric pressure and experimental seizures, animals were tested in decompression chambers in which altitudes of 12,500 ft (474 mm Hg) to 25,000 ft (280 mm Hg) were simulated (4,5). Increases in central nervous system excitability and severity of seizures at these low pressures could be explained by changes induced in tissue CO_2 and O_2. The present study was designed to investigate the effect of relatively small changes in barometric pressure on the threshold to seizures so that results might be related more meaningfully to the variations encountered in the normal environment.

Materials and metrods. A pressure chamber constructed of transparent Plexiglass was fit-

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