THE CULTURE OF DISSOCIATED CELLS FROM RAT CEREBRAL CORTEX

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INTRODUCTION

Development of methods for cultivation of brain tissue in vitro has provided a potentially useful system for the examination of brain metabolism in isolation from the variety of complex physiological events that occur during the perinatal period.

Of the various methods employed in the culture of brain cells, both the Rose perfusion chamber (1), and the Maximow slide assembly (2) do not allow the culture of an amount of tissue sufficient for the fractionation and analysis of lipids, and for any necessary enzymatic studies. In our initial explorations we attempted to circumvent these problems by the use of brain explants derived from frontal lobes of immature rats. These explants were maintained in plastic flasks, using serum-

supplemented medium. Once cellular outgrowth had begun, a lipid-free, chemically defined medium was substituted, and the appropriate metabolic studies were conducted. Under these conditions explants continued to incorporate lipid precursors for some 48 h. However, the area of active metabolism was probably confined to the margins of the explants, and long-term incorporation studies were limited by the transient viability of cultured tissue in a chemically defined medium (3, 4).

In this publication we wish to describe the preparation and maintenance of dissociated cells from cerebral hemispheres of embryos or immature newborn rats. The system of dissociated cell cultures is based on the ability of dissociated cells to reassociate, and to form well organized aggregates in the course of culture, which may acquire some of the characteristics of the differentiated original tissue.

METHODS

Cultivation of Cultures

Cerebral hemispheres from newborn Sprague-Dawley rats, or 14–17 day embryos, were dissected under sterile conditions and placed in a balanced Hanks' salt solution (Grand Island Biological Co., Grand Island, N. Y.). Meninges and blood vessels were carefully removed and tissue was cut into fragments about 0.5 mm in diameter. The total amount of tissue used was approximately 0.5 g wet weight. The fragments were collected, placed in an Erlenmeyer flask with a magnetic stirrer, and 10 ml of an 0.125% trypsin solution (Grand Island Biological Co.) were added. The tissue was incubated for 3–4 min at 37°C, and 10 ml of the nutrient solution were added. The latter was composed of 20% heat-inactivated fetal calf serum (Rehatuin-Reheis Chemical Company, Chicago, Ill.), 20% whole egg ultrafiltrate (Grand Island Biological Co.), in Eagle's Minimal Essential Medium (MEM) (Grand Island Biological Co.), fortified with glucose (600 mg%), and a penicillin-streptomycin solution (0.25 mg each per 100 ml of medium). The suspension was centrifuged at room temperature for 5 min at 700 *g*. The supernate was completely removed and replaced with 5 ml of nutrient solution. The cell suspension was aspirated five to ten times through a fine-mouth Pasteur pipette to accomplish complete dissociation of the tissue. Cells were then transferred into plastic Falcon flasks (T-30 or T-60) (Becton-Dickinson & Co., Rutherford, N. J.) for a final approximate concentration of 2–5 mg wet weight tissue per milliliter of medium. The flasks were incubated at 37°C in an atmosphere of 95% air, 5% CO_2.

Alternatively, cells were dissociated by passing the tissue which had been freed of meninges and blood vessels through a nylon sieve (No. 50, 48 μm mesh) (Trippete and Renaud Co., Paris, France), by the use of gentle pressure applied by means of a rubber policeman. The cells were then collected in the nutrient medium and centrifuged for 5 min at 700 *g*. The sediment was resuspended in the nutrient medium and placed in Falcon flasks as described above. Both methods resulted in a single cell suspension as visualized by phase contrast microscopy.

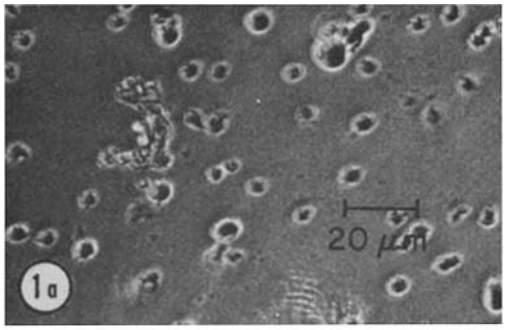
Cells were allowed to aggregate and attach to the floor of the flask for at least 48 h before changing the medium. Thereafter, the medium was changed every 3–4 days, depending on its acidity. Phase contrast microscopy was employed for observation of cultures during their growth period.

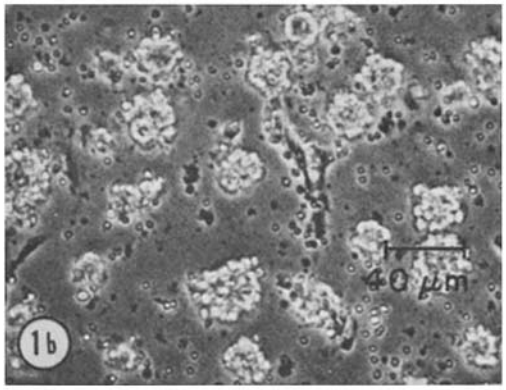
RESULTS

Morphology

Immediately after their dissociation, cells had a spherical and uniform appearance. As depicted in Fig. 1 a, they were small and usually possessed a distinct halo. Within 4–8 h after being placed into the culture flasks, the cells had begun to aggregate, and to attach themselves to the plastic floor in concentric aggregates that varied in size from a few to several hundred cells (Fig. 1 b). Aggregation occurred while cells were still floating in the medium and preceded their attachment to the plastic floor. Attachment was followed by the appearance of cell processes which in many cases grew from one cellular aggregate to another (Fig. 2). After 48–72 h in culture in serum-supplemented medium, these processes (neurites) multiplied and enlarged, and ultimately formed a network that extended throughout the flask. From this time on, some cells could be tentatively identified as neurons or neuroglia. The former were characterized by a prominent nucleus and nucleolus and axon-dendritic processes stainable with silver nitrate (Fig. 3 a). The neuroglia-like cells were small with a dense nucleus (Fig. 3 b). After 72 h in culture, the cell aggregates became increasingly connected by neurites, the latter ultimately arranging themselves into bundles of varying lengths and thickness, which in most instances developed on the surface of the cultures (Fig. 3 b).

Between the second and third week in culture, some processes lost their attachment to the cell aggregrates, ruptured, and their free ends were seen to float in the medium. The cells, however, continued to multiply and develop outgrowths





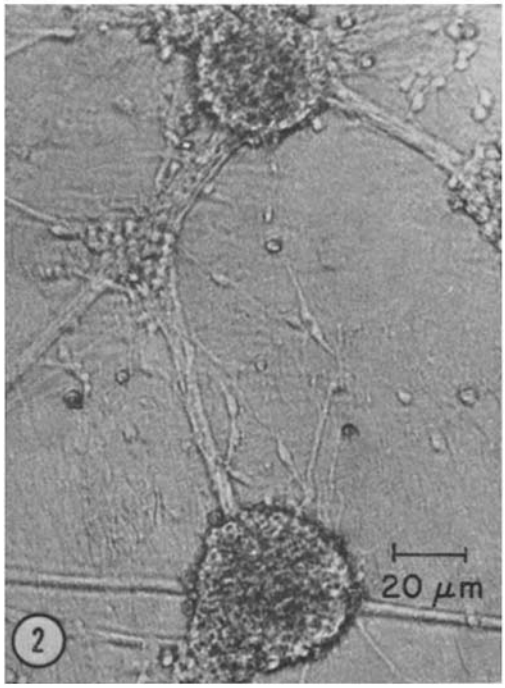


FIGURE 1 a Trypsin dissociated cells from 14–17 day rat embryo cerebral hemispheres 30 min after being placed into culture. Note the uniform appearance of the small cells, and their halo. Phase contrast microscopy. × 250.

from the aggregates even in the absence of fibrous processes. A third type of cell population, consisting of polygonal, ependyma-like cells, failed to aggregate, but ultimately formed a near monolayer, which served as a background for the processes (Fig. 4). After 4 wk in vitro, long processes were no longer apparent, and cultures consisted almost entirely of a confluent layer of ependyma-like cells on top of which neuron- and neuroglia-like cells were seen.

As a rule, cell growth was better in cultures derived from embryos than in those obtained from newborn rats. The former cultures tended to develop an enriched population of oligodendroglia-like and neuron-like cells. The average cell size was smaller than in cultures derived from newborn brain, and continued to remain that way, even up to 28 days in vitro.

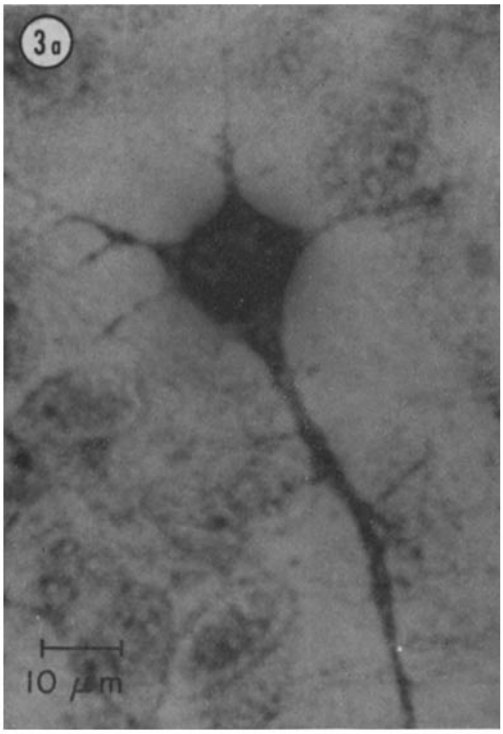
When the dissociated, sieved cells were placed in flasks coated with rat collagen (2), fibrous processes were already apparent after 48 h in vitro, and the number of processes arising from each cell tended to be greater (Figs. 5 a, 5 b). The two methods used for dissociation, sieving and trypsinization, did not affect the appearance of the cultures.

DISCUSSION

Dissociated cells from neural tissues have been used for morphological studies in a number of laboratories. Cavanaugh (5) observed the development of neurons from dissociated chick embryo spinal cord. Varon and Raiborn (6) subsequently cultured chick embryo cells, dissociated by trypsinization or sieving in a plasma clot sys- and found them to retain their ability to grow fibers and to form intercellular contacts. They distinguished three cell types: A cells were multipolar, with an excentrically located nucleus, fairly thick processes, and little mitotic activity. They probably represented neurons and correspond to the neuron-like cells observed in our system (Fig. 3 a). B cells were smaller and

FIGURE 1 b Trypsin dissociated cells from 14–17 day rat embryo cerebral hemispheres, 4–8 h in vitro. Cells have begun to aggregate into clumps. As yet there is no process formation, and no obvious differentiation. Phase contrast microscopy. × 125.

FIGURE 2 Trypsin dissociated cells from 14–17 day rat embryo cerebral hemispheres. 1–2 days in vitro. Aggregation into colonies has been completed, and cell processes extend from one aggregate to another. Phase contrast microscopy. × 250.



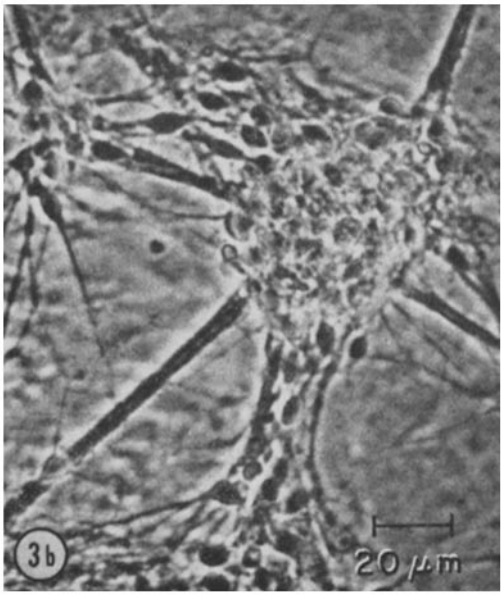


FIGURE 3 a Trypsin dissociated cells from 14–17 day rat embryo cerebral hemispheres, 9 days in vitro. Holmes silver nitrate stain demonstrating argyrophilic neurites extending from a neuron-like perikaryon. × 500.

FIGURE 3 b Trypsin dissociated cells from 14–17 day rat embryo cerebral hemispheres, 10 days in vitro. Bundles of connecting neurites are evident. Many of the cells in the aggregates are small and dark, and may be of glial nature. Phase contrast microscopy. × 250

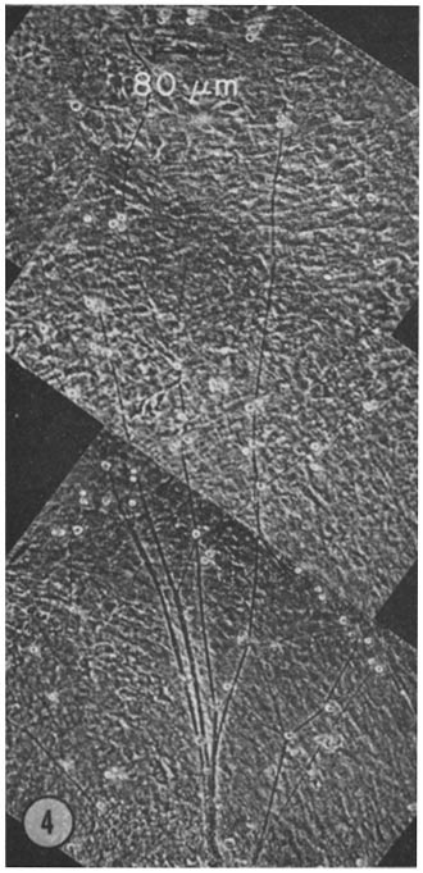
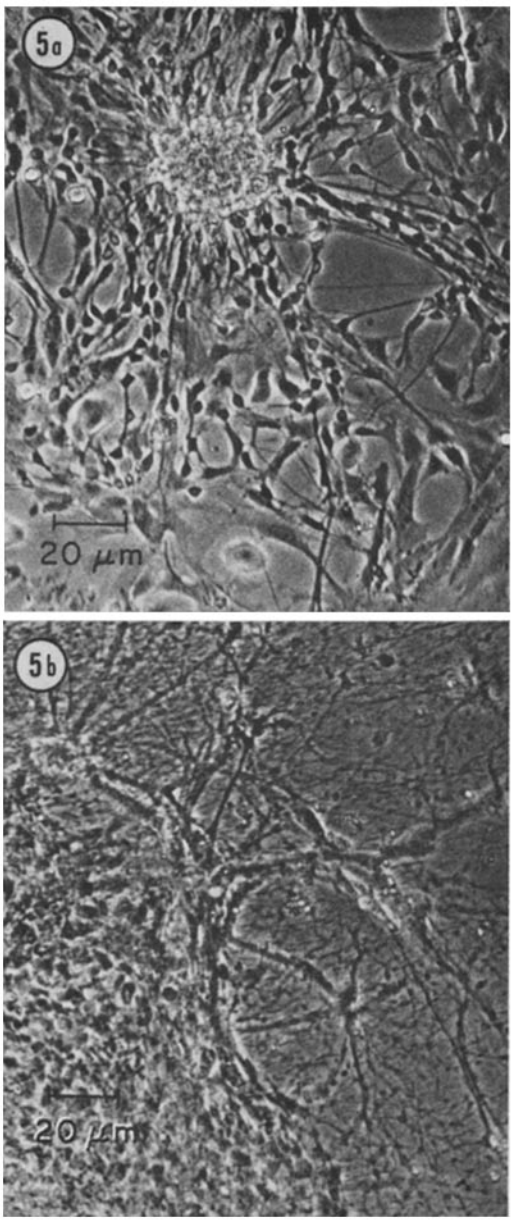


FIGURE 4 Trypsin dissociated cells from 14–17 day rat embryo cerebral hemispheres, 11 days in vitro. Montage showing the extent of long, slender fibers against a background matrix of polygonal cells. Phase contrast microscopy. × 65.

denser, with bipolar or pseudounipolar processes, and they were also unable to proliferate. Varon and Raiborn suggested that they represented immature neurons, or oligodendroglia. They correspond to the small, dense, glia-like cells observed by us to constitute a considerable portion of the cellular aggregates (Fig. 3 b). C cells were large, oval, with a central nucleus, a clear cytoplasm, and gross processes. They undoubtedly correspond to our polygonal cells (Fig. 4). This last cell type ultimately became dominant in long-term cultivated cultures (6, 7).

Bornstein and Model (8) extended these studies



FIGURES 5 a and 5 b Trypsin dissociated cells from 14–17 day rat embryo cerebral hemispheres grown on plastic (Fig. 5 a) or collagen-coated (Fig. 5 b) flasks. 13 days in vitro. Note the ramification of processes in the collagen grown cultures as compared with those grown on plastic. Phase contrast microscopy. × 125.

to dissociated embryonic mouse cerebral cortex, brain stem, and spinal cord. As in the avian embryo, no distinction between cell types was observed during the first few days in vitro. Subsequently, as demonstrated by electron microscope examination, cultures form nerve processes, and axosomatic and axodendritic synapses. Crain and Bornstein (9) and Fischbach (10) have recorded bioelectrical activities from cultures derived from mammalian and avian embryos, respectively, and have demonstrated propagation of neuronal impulses and synaptic transmission. Enzymatic and biochemical changes that accompany growth and differentiation of dissociated embryonic mouse brain cells parallel the biochemical maturation of the brain (11, 12).

The results of extensive metabolic studies on cultured dissociated brain cells, prepared according to methods described in this publication, are being reported elsewhere[1].

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REFERENCES

1. ROSE, G. 1954. A separable and multipurpose tissue culture chamber. *Tex. Rep. Biol. Med.* **12**:1074.
2. BORNSTEIN, M. B. 1958. Reconstituted rat-tail collagen used as substrate for tissue cultures on cover slips in Maximow slides and roller tubes. *Lab. Invest.* **7**:134.
3. MENKES, J. H. 1971. Lipid metabolism in brain tissue explants. *J. Neurochem.* **18**:1433.
4. MENKES, J. H. 1972. Lipid metabolism of brain tissue in culture. *Lipids.* **7**:135.
5. CAVANAUGH, M. W. 1955. Neuron development from trypsin-dissociated cells of differentiated spinal cord of the chick embryo. *Exp. Cell Res.* **9**:42.
6. VARON, S., and C. W. RAIBORN. 1969. Dissociation, fractionation, and culture of embryonic brain cells. *Brain Res.* **12**:180.
7. SENSENBRENNER, M., J. BOOHER, and P. MANDEL. 1971. Cultivation and growth of dissociated neurons from chick embryo cerebral cortex in the presence of different substrates. *Z. Zellforsch. Mikrosk. Anat.* **117**:559.

[1] Yavin, E., and J. H. Menkes. Glyceride metabolism in cultured dissociated cells. Submitted to *J. Neurochem.*

8. BORNSTEIN, M. B., and P. G. MODEL. 1972. Development of synapses and myelin in cultures of dissociated embryonic mouse spinal cord, medulla and cerebrum. *Brain Res.* **37**:287.
9. CRAIN, S. M., and M. B. BORNSTEIN. 1972. Organotypic bioelectric activity in cultured reaggregates of dissociated rodent brain cells. *Science* (*Wash. D. C.*). **176**:182.
10. FISCHBACH, G. D. 1970. Synaptic potentials recorded in cell cultures of nerve and muscle. *Science* (*Wash. D. C.*). **169**:1331.
11. WILSON, S. H., B. K. SCHRIER, J. L. FARBER, E. J. THOMPSON, R. N. ROSENBERG, A. J. BLUME, and M. W. NIRENBERG. 1972. Markers for gene expression in cultured cells from the nervous system. *J. Biol. Chem.* **247**:3159.
12. SEEDS, N. W. 1971. Biochemical differentiation in reaggregating brain cell culture. *Proc. Natl. Acad. Sci. U. S. A.* **68**:1858.