New-Forming Retinal Synapses in vitro

Since HARRISON's[1] investigations the neoformation in vitro of neuronal interconnections was presumed to exist, but the subsequent numerous observations by LEVI[2] gave evidence of only transitory contacts. Later, synaptic connections between neurons were demonstrated in explants of central nervous tissue maintained in culture even for months. But it could not be determined so far whether these synapses were predifferentiated or predetermined structures brought into the cultures and only maintained, or whether they were newly established in the cultivated explants or in the peripheral zone of outgrowing fibres.

The results obtained by CALLAS and HILD[3], HILD and TASAKI[4], HILD[5], and WOLF[6] are more consistent with the former view, either because of the age of the animals from which the explants were obtained (new-born rats, where synaptic structures had already differentiated), or because of the techniques employed.

BUNGE et al.[7], using spinal cord explants removed from rat fetuses, noticed a greater number of synapses in long-term cultures than at the explantation time, and a neuropile which had formed ex-novo on the cut surface of the spinal cord fragment. Anyway it might be only the result of a further differentiation of already established interrelationships among cells in vivo.

The question of the functional activity of these connections observed in vitro was raised, and a correlation between microscopically recognizable synaptic structures and their electrophysiological behaviour became necessary.

CRAIN[8], through recordings from cultured spinal ganglion cells, revealed important similarities of bioelectrical behaviour with various types of neurons in situ, even if he could not detect significant differences as a function of the cultures' age, demonstrating that 'cultured cells could retain at least many of their characteristic functions after long periods of isolation in vitro'. Similarly HILD and TASAKI[4] demonstrated that cerebellar neurons had repetitive spontaneous discharges, but they failed to find any evidence of neuronal interactions; and recently HILD[5] concluded that in his cultures neurons no longer had synaptic connections with other neurons, and if there were patterns of neuronal circuits they were remnants of the originally larger circuits present in situ. This phenomenon of survival of non-functioning structures is not improbable, bearing in mind that the preservation of the synaptic apparatus was demonstrated in Ichtyopsida (f.i. in the physiological degeneration of the Mauthner cells, STEFANELLI[9]).

[1] R. G. HARRISON, Proc. Soc. exp. Biol. Med. *4*, 140 (1907).
[2] G. LEVI, Archs Biol., Paris *72*, 133 (1941).
[3] G. CALLAS and W. HILD, Z. Zellforsch. mikrosk. Anat. *63*, 686 (1964).
[4] W. HILD and I. TASAKI, J. Neurophysiol. *25*, 277 (1962).
[5] W. HILD, Z. Zellforsch. mikrosk. Anat. *69*, 155 (1966).
[6] M. K. WOLF, J. Cell. Biol. *22*, 259 (1964).
[7] R. P. BUNGE, M. B. BUNGE and E. R. PETERSON, J. Cell. Biol. *24*, 163 (1965).
[8] S. M. CRAIN, J. comp. Neurol. *104*, 285 (1956).
[9] A. STEFANELLI, Experientia *9*, 277 (1953).

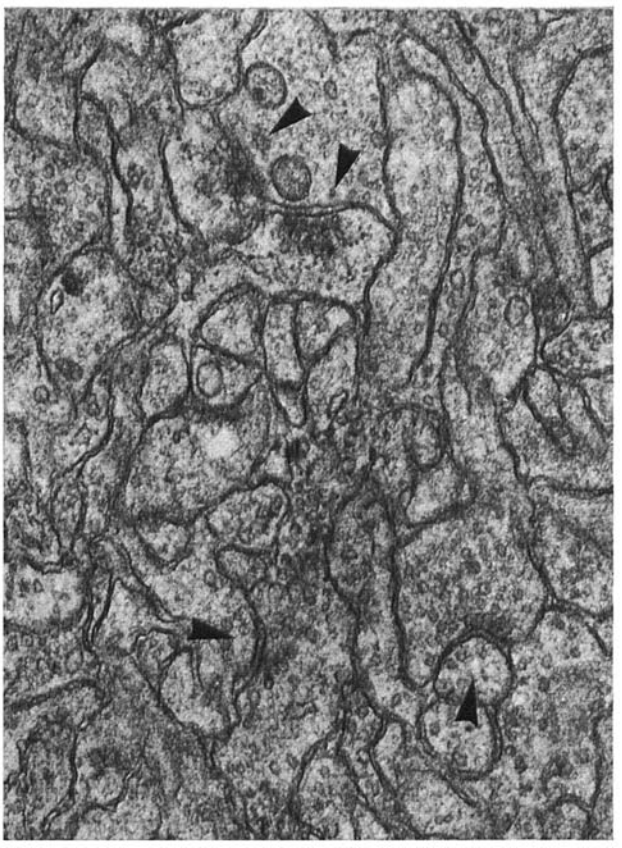

Fig. 1. Re-aggregated retina of chick after 25 days of culture (disaggregated at 4 days). Note synaptic junction of neuropile.

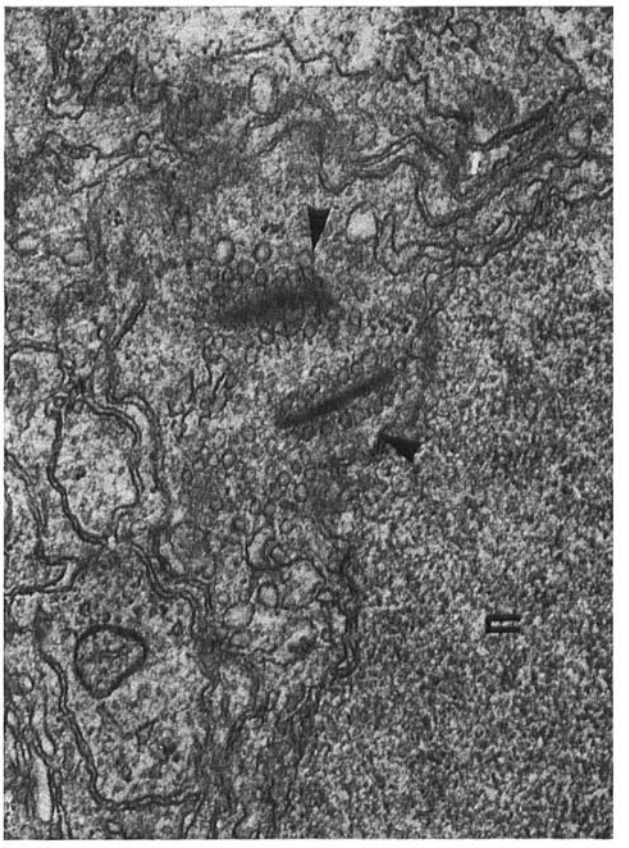

Fig. 2. Synaptic segment of photoreceptor of retina disaggregated at 4 days and cultivated after re-aggregation for 25 days. Note synaptic ribbons.

The experiments of CRAIN and PETERSON[10] and PETERSON et al.[11], through microelectrophysiological stimulation and recording techniques, indicate that neurons, if properly maintained in vitro, reveal a complex bioelectric activity resembling that of synaptic networks in vivo. It must be considered that synapses were already present at explantation of the nervous tissue, but the authors do not exclude the possibility that additional synapses developed in culture.

The evident difference of opinions on such an interesting problem led us to repeat and carry on the researches already made by some of us (STEFANELLI et al.[12]), trying to obtain long-term cultures and using the electron microscope for the specimen investigations. To overcome the difficult and undecided question of the pre-existence or neoformation of synaptic connections in vitro, we used eye cups of chicken embryos aged 4 days, disaggregated by trypsin; in such a way we dealt with a material that in vivo was still undifferentiated as far as nervous elements and neuronal interconnections. We chose the eye cup because, among the retinal layers, the synaptic types are morphologically well identifiable. The disaggregated cells were reaggregated as little balls of about 2 mm and cultured in rolling tubes. Our observations apply to cell aggregates maintained in culture for 25 days. The material was fixed and embedded for optical and electron microscopy.

We could show the differentiation of 3 cellular types: the Müller support cells, identifiable by their gliofilaments, by the microvilli and by the terminal bar at their free surface; the neurons, particularly rich in ribosomes and endoplasmic reticulum, but not distinguishable in bipolar, ganglion and amacrine cells; and lastly the photoreceptors, rods and cones not recognizable from each other.

Recently, EVANS[13] found that the distinctive feature between the two kinds of receptors in the chick retina lies in the presynaptic vesicles, which are rarer and smaller in the cones. But this peculiarity of the cones is not significant enough, and we failed to reveal any difference in our specimens; on the other hand, we never observed the characteristic pattern of double-membrane discs arranged to form a pile of the outer rod segments.

Furthermore, a thick neuropile with numerous synaptic connections showing the peculiar thickening of the membranes and the vesicles at the presynaptic side, was clearly differentiated (Figure 1, arrows).

A very interesting finding was the presence of 'synaptic ribbons', characteristic in the presynaptic sides of the contacts between photoreceptors and dendrites of the bipolar cells (Figure 2, arrows). These synaptic ribbons are common to rods and cones, therefore they could not be of a differential character. We think that our observations provide a valuable morphological basis for the future electrophysiologic verification of the synaptic connections established in vitro.

Riassunto. Cellule di abbozzo oculare di embrione di pollo di 4 giorni di incubazione sono state disgregate, riaggregate e coltivate in vitro per 25 giorni. Al m.e. è risultato un fitto neuropilo ricco di sinapsi tra cui i «synaptic ribbons» caratteristici delle cellule fotorecettrici. Questi risultati sono una prima sicura documentazione di sinapsi formate ex novo in vitro con nuova associazione di cellule.

A. STEFANELLI, A. M. ZACCHEI, S. CARAVITA, A. CATALDI, and L. A. IERADI

Centro di Neuroembriologia del Consiglio Nazionale delle Ricerche, Istituto di Anatomia Comparata della Università di Roma (Italia), 14th July 1966.

[10] S. M. CRAIN and E. R. PETERSON, J. cell. comp. Physiol. *64*, 1 (1964).
[11] E. R. PETERSON, S. M. CRAIN and M. R. MURRAY, Z. Zellforsch. mikrosk. Anat. *66*, 130 (1965).
[12] A. STEFANELLI, A. M. ZACCHEI and V. CECCHERINI, Atti Accad. naz. Lincei Rc. *30*, 818 (1961); Acta Embryol. Morph. exp. *4*, 47 (1961).
[13] E. M. EVANS, Z. Zellforsch. mikrosk. Anat. *71*, 499 (1966).

The Influence of Deprivation of Paradoxical Sleep on Glycogen Content in Various Brain Structures of the Cat

The role of carbohydrate metabolism in brain function has been emphasized many times. Brain tissue has exceptionally great potential and capacity to metabolize glucose, which represents one of its most important energy sources[1]. Glycogen, free and bound (residual), is an integral part of neuronal structure, and suffers considerable changes and quantitative variations dependent upon the functional state of the brain tissue. These variations are most intensive in structures which have the biggest metabolic rate[2]. Recent sleep studies point out the metabolic and enzymatic nature of the mechanism of sleep, especially its paradoxical phase (PS). Our previous experiments have shown that PS deprivation in cats leads to considerable changes in concentration of GABA-GA-AA and that these changes have very specific and selective regional character, limited to certain brain structures[3]. Starting from the fact that the quantity of glycogen reflects the metabolic and at the same time the functional state of the CNS, and that PS deprivation is a specific phenomenon causing prolongation of the paradoxical phase of sleep in the recuperative period, we considered it of interest to examine the quantitative changes of glycogen (total, free and bound) in various brain structures in order to find out the possible correlation between the regional metabolism of glycogen and structures exhibiting neurophysiological evidence for involvement in PS mechanism.

Experiments were carried out on 12 adult cats. 6 cats served as control, while the other 6 were submitted to selective and instrumental PS deprivation[4] for 96 h before being sacrificed. Quick decapitation was done by a

[1] R. BALÁZS and D. RICHTER, in *Regional Biochemistry* (Ed. S. S. VETY and J. ELKES; Pergamon Press, Oxford, London, New York, Paris 1961), p. 49.
[2] A. CHESLER and H. E. HIMWICH, Archs Neurol. Psychiat., Chicago *52*, 114 (1944).
[3] D. MIĆIĆ, V. KARADŽIĆ and LJ. RAKIĆ, Nature, in press (1966).
[4] D. JOUVET, P. VIMONT, J. F, DELORME and M. JOUVET, C. r. Séanc. Soc. Biol. *158*, 756 (1964).