PRESENCE OF TAILED, ASYMMETRIC FORMS OF ACETYLCHOLINESTERASE IN THE CENTRAL NERVOUS SYSTEM OF VERTEBRATES

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1. Introduction

Multiple molecular forms of acetylcholinesterase (acetylcholine hydrolase EC 3.1.1.7) (AChE) have been found in tissues from many sources (vertebrate and invertebrate), in neuronal and non-neuronal cells. They were first shown in electric organs of electric fish [1] as globular as well as tailed asymmetric forms [2–4]. They were then identified in mammalian [5–7] and avian muscle and brain [8,9] and sympathetic ganglia [10,11]. The fast-sedimenting, high M_r forms of AChE or tailed forms [2,12–14], also refered to as A forms [15] have not been found, or demonstrated conclusively, in the vertebrate central nervous system [6,8,16–18], although it was reported that collagenase, which can degrade the tail part of AChE [15,19,20,21] releases two fast-sedimenting AChE forms from chick optic tectum, similar to those obtained by collagenase treatment of the muscle tailed forms [22]. Furthermore, it has been found [18] that the tailed forms of AChE were absent even in the (rat) caudate nucleus, a structure which is known to have the highest AChE content and a high level of cholinergic activity. In rat brain extracts, we have shown that addition of exogeneous muscle tailed forms did not result in their proteolytic degradation [6], which excludes any endogenous degradation process accounting for the non-detection of the tailed forms.

However, we now better understand the regulation of the biosynthesis of the tailed forms (and especially the 16 S AChE in mammalian tissue) which seems to be dependent on muscle or nerve ion-flux-dependent activities [23,24]. The tailed forms can be found in muscle cells cultured alone, or in cultured sympathetic-like transformed cells (PC12), provided they generate spontaneous action potentials. They are not restricted to (or dependent only upon) cholinergic synaptic contacts, as earlier found for the rat adult neuromuscular junction [7]. Thus, they potentially could be found in any active neuron. It becomes an intriguing fact that they were not detected in the CNS tissues so far examined, and it is an important matter to investigate in more detail the possibility of their presence in specific regions of the brain or in different neuronal cell types.

This paper reports the results of a study on mouse, rat and chick brain, or regions of brain, and provides direct evidence for the presence of tailed forms of AChE in a few of these tissues.

2. Materials and methods

Two month old rats (Charles River, France), and mice (129 ReJ stain, from our own breeding colony) and white Leghorn-strain chicks (6 week old, CEA, Saclay) or embryos (at 19 days gestation) were used in this study. The eggs were supplied by an industrial hatchery after 11 days incubation and incubation was continued in a Felmon incubator. Mice and rats were kept on a 14 h light–10 h dark schedule and were sacrificed in the afternoon to avoid changes due to circadian rhythms [25]. Rats and mice were sacrificed by ether exposure and chicks by decapitation. The brains were dissected over ice to isolate the cerebellum, cortex, caudate nucleus, substantia nigra and

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for the chick, the optic lobes and brain stem. The tissues were homogenized in 20 vol. 10 mM Tris–HCl (pH 7.2), 1% Triton X-100, 1 M NaCl and 1 mM EGTA (homogenization buffer), in a glass–glass conical homogenizer. Sometimes an 'anti-protease medium' was used to test the possibility of some artifactual proteolytic process after homogenization [24], but gave no substantial difference from the standard medium in the analyses detailed below. The resulting homogenate was centrifuged at 20 000 × g for 15 min, in a Sorvall RC-2 refrigerated centrifuge (4°C), in 1 ml plastic tubes. Supernatants were used for further biochemical analysis. Acetylcholinesterase activity was estimated in triplicate as in [26], using acetylthiocholine iodide as a substrate, and protein concentration by an adaptation of the procedure in [27], to eliminate flocculating material.

Sedimentation analysis of acetylcholinesterase was essentially performed as in [24]. Briefly, aliquots of homogenates (75 μl), were loaded onto pre-formed continuous sucrose gradients (5–20%, SW 41 Beckman rotor and L 8 Beckman ultracentrifuge, 15 h, 4°C, 38 000 rev./min). Fractions were collected, assayed for AChE activity and sedimentation coefficients were estimated by comparison with enzyme markers (β-galactosidase $s_{20,w}$ 16 S; catalase $s_{20,w}$ 11.4 S; and horse liver alcohol dehydrogenase $s_{20,w}$ 7.4 S). The contribution of non-specific cholinesterases to acetylthiocholine hydrolysis pattens was found to be negligible in our experimental condition after specific irreversible inhibition of acetylcholinesterase by 10^{-8} M methyl phosphonothioate [28]. Ethylene glycol-bis-(β-amino-ethyl ether) N,N'-tetra acetic acid (EGTA), acetylthiocholine iodide, 5,5′-dithio (bis)-dinitrobenzoic acid, β-galactosidase horse liver alcohol dehydrogenase were from Sigma and catalase from Boehringer. Ethyl-S-(2 (bis(1-methylethyl)amino)-ethyl) methylphosphonotioate was a gift from Dr F. Leterrier (Hospital Percy, Clamart).

3. Results

3.1. *Mammalian central nervous system*

The homogenization of 2 month old rat or mouse brains, using an homogenization medium containing high-ionic strength buffer, Triton X-100, and EGTA results in the solubilization of ~95% of the brain total AChE content (as estimated by successive homogenizations of the same tissue and resulting pellets).

The specific activities of AChE are of the order of 57 (rat) and 46 (mouse) $\Delta A \cdot h^{-1} \cdot$ mg protein^{-1}. Sedimentation analysis shows that AChE is present predominantly as a 10 S form, and in a minor proportion as a '4 S' form; in agreement with [6,18] (fig.1) and no '16 S' form can be detected. However rat and more clearly, mouse cerebellar homogenates contain detectable levels of '16 S' AChE, as shown on fig.1, up to 2–3% in mouse cerebellum. Essentially the same results were obtained with 6 month old animals. If the fractions are incubated in Ellman's enzymatic assay mixture for much longer periods of time, in order to get a maximal absorbance variation in the '16 S' region (up to 15-times longer than the usual incubation times used to obtain a maximal absorbance variation of 1.5 at the major 10 S peak) it becomes evident that the mouse cerebellum contains a higher proportion of '16 S' AChE than rat cerebellum (fig.3). It is worth noting that total AChE specific activities

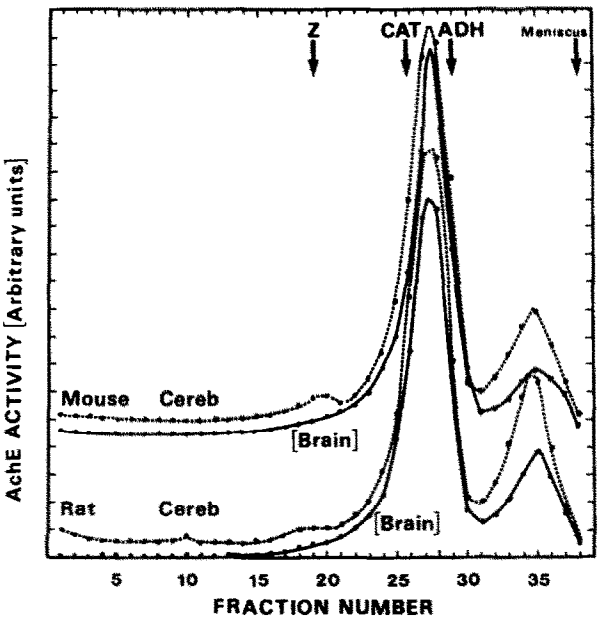

Fig.1. The multiple molecular forms of acetylcholinesterase in mouse and rat brain: presence of the '16 S' tailed form in mouse and rat cerebellum. Sedimentation is from right to left. Ordinates are given in arbitrary units (ΔA_{412}). However, the sedimentation profiles corresponding to rat and mouse (•—•) brains are directly comparable and the same is true for rat and mouse (· · ·) cerebella (Cereb.) AChE profiles. The cerebellum fractions have been incubated 4-times longer than the brain fractions. Sedimentation markers are β-galactosidase (Z, 16 S) catalase (CAT.11.4. S) and alcohol dehydrogenase (ADH 7.4 S).

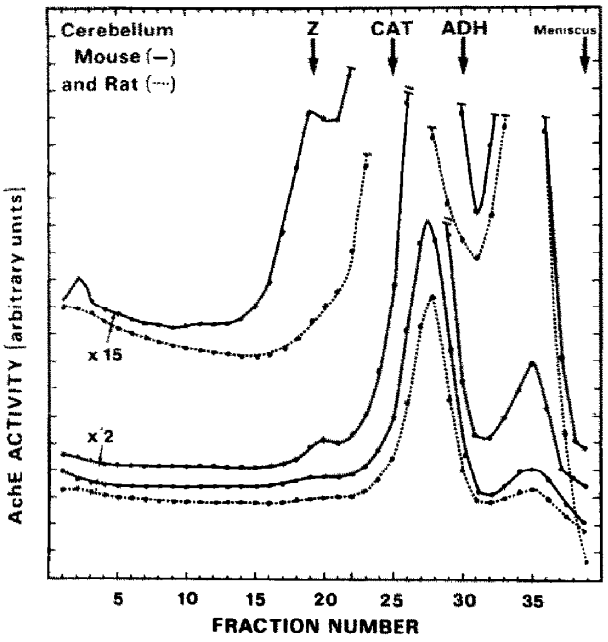

Fig.2. Comparison between the rat and mouse cerebella for their content in 16 S tailed form. Same technique as in fig.1 but AChE profiles have been incubated for usual periods of time (rat 2 h (· · ·) and mouse 1 h (·——·)) or longer ones (mouse: 2-times more than usual (× 2); 15-times more (× 15); and rat 15-times more (× 15).

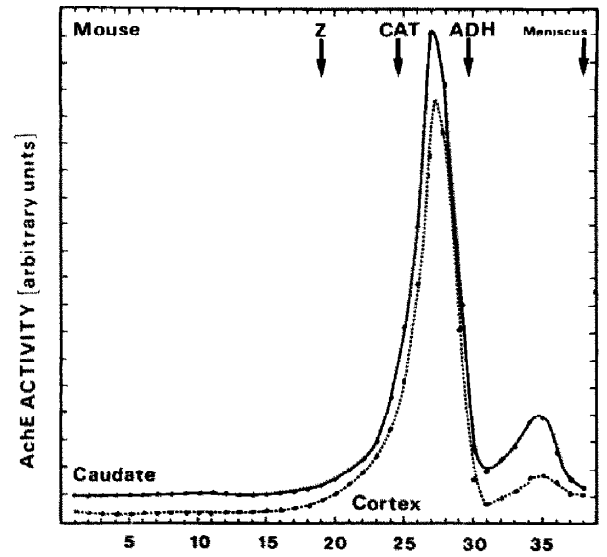

Fig.3. Lack of '16 S' tailed form in mouse caudate nucleus and cortex. Same technique as in fig.1. Cortex AChE profile may be compared to the profiles in fig.2 (same ordinates, same 'usual' incubation time), when the caudate nucleus profile has been incubated 5-times less.

are slightly higher in the rat cerebellum homogenate (19 $\Delta A \cdot h^{-1} \cdot mg^{-1}$) than in the mouse cerebellum homogenate (13 $\Delta A \cdot h^{-1} \cdot mg^{-1}$). The precise sedimentation coefficients of the main molecular forms of rat and mouse AChE have been determined by comparison with the sedimentation marker enzymes and are listed in table 1. We did not find detectable levels of '16 S' in caudate nucleus, cortex or substantia nigra either in rat or in mouse, as illustrated for mouse in fig.3.

3.2. *Avian central nervous system*

We homogenized under the same conditions as for rat and mouse brain tissues, several chick brain regions. The solubilization reached after one homogenizing step, is of the order of 95–97% of the total AChE activity (as estimated after successive homogenizations) in 6 week old cerebellum (254 $\Delta A \cdot h^{-1} \cdot mg^{-1}$), cortex (192 $\Delta A \cdot h^{-1} \cdot mg^{-1}$) brain stem (178 $\Delta A \cdot h^{-1} \cdot mg^{-1}$) and optic lobes (300 $\Delta A \cdot h^{-1} \cdot mg^{-1}$).

In chick brain the tailed forms of AChE are present in few regions although they are generally difficult to detect: the optic lobes are one of the richest source of '20 S' AChE, in 6 week old chick (fig.4) but long

Table 1
The multiple molecular forms of acetylcholinesterase and their sedimentation coefficients in the mammalian and avian central nervous system

	Fast-sedimenting tailed forms	Other forms
Mouse	16 S ± 0.12 (n = 4)	9.47 S ± 0.08 (n = 10) 3.48 S ± 0.04 (n = 11)
Rat	16.1 S (mean of 2 det.)	9.42 S ± 0.09 (n = 11) 3.65 S ± 0.11 (n = 18)
Chick	25.5 S ± 0.23 (n = 12) 21.13 S ± 0.13 (n = 13)	11.6 S ± 0.07 (n = 9) 7.03 S ± 0.08 (n = 12) 4.7 S ± 0.01 (n = 7)

The sedimentation coefficients were determined in the presence of 1 M NaCl, 1 mM EGTA, 1% Triton X-100 and 10 mM Tris–HCl (pH 7.2). Standard errors are given and n is the number of independent determinations. The mammalian fast sedimenting, tailed form (16 S) is only present in cerebellum (but chick tailed forms were not found in chick cerebellum). For rat, they were only detected, at significant levels, in 2 expt. out of 5, and the given sedimentation coefficient is consequently the mean value of only 2 determinations. For mouse cerebellum, the '16 S' AChE was present in all 4 expt. performed. All other forms were present in all tissues examined

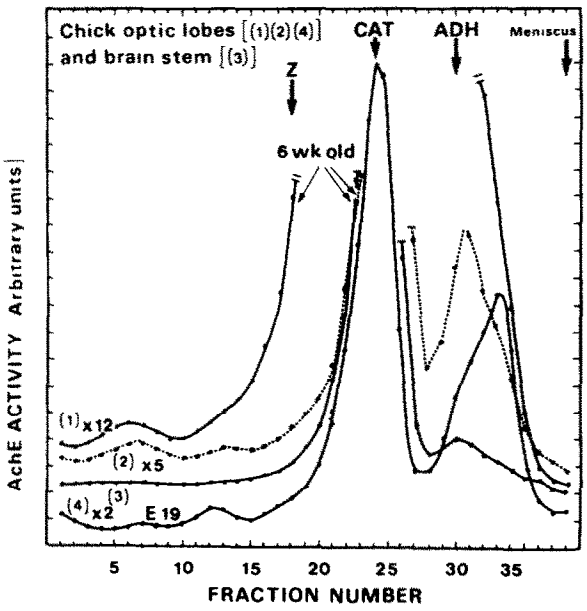

Fig.4. The tailed forms of AChE in chick optic lobes and brain stem. Same technique as in fig.1. Chick embryo optic lobes (E 19) were incubated 2-times more (·—·; × 2) and 12 times more (·—·; × 12) than 6 week old chick optic lobes (7 min. incubation; ·——·), when brain stem fractions were incubated 5-times more (· · ·; × 5).

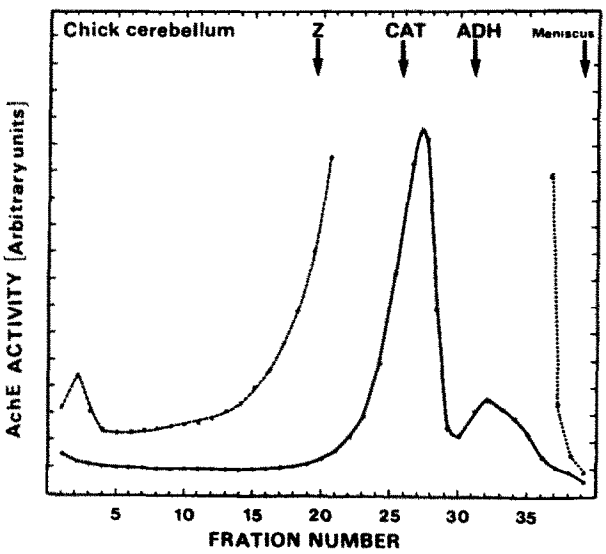

Fig.5. No tailed form in 6 week old chick cerebellum. Same technique as in fig.1 and same incubation time (·—·) as for 6 week old optic lobes AChE in fig.4. The fractions have also been incubated 20-times more (⊙——⊙), to show that even reaching the limit of sensitivity of our method, the tailed forms cannot be shown in chick cerebellum.

incubations are necessary to demonstrate it (12-times more than the usual incubation time), but only as a shoulder to the main 11 S peak. However, an additional form (25.5 S, designated as the 26 S AChE, table 1) is more readily detected. To our knowledge, the occurence of this form has not yet been reported. Both the '20 S' and '26 S' forms are demonstrated, for shorter incubation times in brain stem homogenates (fig.4). It is in the chick embryo, just before hatching (E19) that the highest levels of '20 S' and '26 S' are found in the optic lobes ('26 S' AChE is in higher amounts than '20 S'). These forms are not detected in 6 week old chicks cerebellum (fig.5), in our experimental conditions, even with the longest period of incubation.

4. Discussion

The previously reported failure to detect tailed forms of AChE in the central nervous system was rather puzzling in view of the diversity of neuronal cells and their state of differentiation and electrical activity. However, this same diversity may allow us to think that CNS cells, which are able to synthesize AChE tailed forms (if they exist), could represent a very minor proportion of the general cell population. The use of EGTA and Triton X-100 together with high-ionic strength buffer allowed us to directly demonstrate the presence of tailed AChE forms, essentially in the mammalian cerebellum. The mouse cerebellum is a better source than the rat in our conditions. We also found the tailed forms of AChE in diverse brain regions in the chick, particularly in the optic lobes.

The homogenization of rat or mouse whole brains probably dilutes the amount of solubilized tailed forms to a point where they become no longer detectable by our techniques. It is worth noting that the structure of the mammalian brain which contains the highest amounts of ACh activity and shows a high level of cholinergic activity, the caudate nucleus, has no detectable level of tailed forms of AChE. This may be due to the fact that most or all neurons in this structure are not in a differentiation state allowing the synthesis of these forms. But it may also be due to a lack of accumulation of significant levels of these forms. This could happen if there is a very active secretion of these forms [16] or few of the very differentiated which areas can accumulate these

forms, such as synaptic contacts (these are very small areas, and they are probably the only sites of accumulation, as in adult mammalian neuromuscular junctions [29]).

Important steps of differentiation are often accompanied by sharp changes in the presence or amount of some of the AChE forms. For example, synapse formation in vivo (rat muscle) is accompanied by the induction of '16 S' AChE [7]; the '10 S' AChE becomes predominant in rat brain during post-natal maturation [6], whereas the rise of 10 S in cerebellum correlates well with the differentiation of Purkinje cells and the external granular layer [18]; chick '11 S' AChE increases in sympathetic ganglia after hatching, when cytological maturity has been reached but junctional synaptic activity is still increasing [11]. The tailed forms (mainly rat or mouse 16 S and chick 20 S), in vivo, in adult vertebrates (rat and chicks) muscles, disappear upon denervation [5,6,8,30], but remain in the denervated rat superior cervical ganglion [10] and are present in rat muscle cells cultured alone [24] or cultured NGF-treated pheochromocytoma cells (PC 12) [23], when action potentials can be generated. It thus seems that the presence of the tailed forms of AChE is more directly related to muscle or nerve activity, or excitability, than to a direct nerve effect and could be an index of differentiation of these cells.

The direct solubilization of the tailed forms from mouse, rat and chick brain tissues definitely prove the proposition in [22] of their existence in chick central nervous system, based on the indirect evidence of collagenase solubilization of fast-sedimenting AChE forms. The failure of their procedure to directly solubilize the tailed forms is due, in our opinion, to the absence of EGTA in the homogenization medium. We have shown on other tissues (mouse mucles and cultured muscle cells) that EGTA increases the solubilization yield of the tailed forms (F. R., in preparation). We also have preliminary evidence for the presence of a 17.6 S tailed form in the frog central nervous system (M. N., F. R., in preparation): optic tectum, cerebellum, telencephalon and spinal cord.

The fact that, in mammalian brain, the tailed forms are found in the highest amount in cerebellum, in spite of very little apparent cholinergic transmission and low content in choline acetyltransferase [31,32] cannot be explained at present, nor can their absence, in the limits of our methods, in chick cerebellum. In mammalian cerebellum, AChE is not found in adult Purkinje cells [33], but may be localized in the granular layer (terminals of the mossy and climbing fibers), the Golgi cells and the deep nuclei [34,35]. The mossy and climbing fiber terminals, and the corresponding inter-cellular clefts, are possibly one of the preferential locations of the tailed forms.

The presence of the tailed forms in the central nervous system opens up new perspectives in studying its development and it will be interesting to determine which cells and structures possess these forms. The question of their physiological function is most interesting, especially in view of the fact that at the level of the neuromuscular junction, these forms are believed to play a predominant role in terminating the action of the neurotransmitter [29]. A more extensive study of other brain regions and in other species is under way.

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References

[1] Massoulié, J. and Rieger, F. (1969) Eur. J. Biochem. 11, 441–445.
[2] Rieger, F., Bon, S., Massoulié, J. and Cartaud, J. (1973) Eur. J. Biochem. 34, 539–547.
[3] Bon, S., Rieger, F. and Massoulié, J. (1973) Eur. J. Biochem. 35, 372–379.
[4] Dudaï, Y., Herzberg, M. and Silman, I. (1973) Proc. Natl. Acad. Sci. USA 70, 2473–2476.
[5] Hall, Z. W. (1973) J. Neurobiol. 4, 343–361.
[6] Rieger, F. and Vigny, M. (1976) J. Neurochem. 27, 121–129.
[7] Vigny, M., Koenig, J. and Rieger, F. (1976) J. Neurochem. 27, 1347–1353.
[8] Vigny, M., Di Giamberardino, L., Couraud, J. Y., Rieger, F. and Koenig, J. (1976) FEBS Lett. 69, 277–280.
[9] Marchand, A., Chapouthier, G. and Massoulié, J. (1977) FEBS Lett. 78, 233–236.
[10] Gisiger, V., Vigny, M., Gautron, J. and Rieger, F. (1978) J. Neurochem. 30, 501–516.

[11] Taylor, P., Rieger, F. and Green, L. A., (1980) Brain Res. 182, 383–396.
[12] Cartaud, J., Rieger, F., Bon, S. and Massoulié, J. (1975) Brain Res. 88, 127–130.
[13] Bon, S., Huet, M., Lemonnier, M., Rieger, F. and Massoulié, J. (1976) Eur. J. Biochem. 68, 523–530.
[14] Rieger, F., Bon, S., Massoulié, J., Cartaud, J., Picard, B. and Benda, P. (1976) Eur. J. Biochem. 68, 513–521.
[15] Bon, S., Vigny, M. and Massoulié, J. (1979) Proc. Natl. Acad. Sci. USA 76, 2546–2550.
[16] Fernandez, H. L., Duell, M. J. and Festoff, B. W. (1979) J. Neurochem. 32, 581–585.
[17] Rotundo, R. L. and Fambrough, D. M. (1979) J. Biol. Chem. 254, 4790–4799.
[18] Wade, P. D. and Timiras, P. S. (1980) Dev. Neurosci. 3, 101–108.
[19] Rieger, F., Bon, S. and Massoulié, J. (1973) FEBS Lett. 36, 12–16.
[20] Lwebuga-Mukasa, J. S., Lappi, S. and Taylor, P. (1976) Biochemistry 15, 1425–1434.
[21] Rieger, F., Ruberg, M. and Shelanski, M. L. (1979) Brain Res. 170, 568–571.
[22] Villafruela, M. J., Barat, A., Villa, S. and Ramirez, G. (1980) FEBS Lett. 110, 91–95.
[23] Rieger, F., Greene, L. A. and Shelanski, M. L. (1980) Dev. Biol. 76, 238–243.
[24] Rieger, F., Koenig, J. and Vigny, M. (1980) Dev. Biol. 76, 358–365.
[25] Moudgil, V. K. and Kanungo, M. S. (1973) Comp. Gen. Pharmacol. 4, 127.
[26] Ellman, G. L., Courtney, K. D., Andres, V. and Featherstone, R. M. (1961) Biochem. Pharmacol. 7, 88–95.
[27] Lowry, O. H., Rosebrough, M. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[28] Vigny, M., Bon, S., Massoulié, J. and Leterrier, F. (1978) Eur. J. Biochem. 85, 317–323.
[29] Koenig, J. and Rieger, F. (1980) Dev. Neurosci. in press.
[30] Koenig, J., Bournaud, R. and Rieger, F. (1980) in: Ontogenesis and functional mechanisms of peripheral synapses, (Taxi, J. ed) pp. 313–325, Elsevier/North-Holland, Amsterdam, New York.
[31] Silver, A. (1967) Int. Rev. Neurobiol. 10, 57–109.
[32] Valcana, T., Lias, C. and Timiras, P. S. (1974) Brain Res. 73, 105–120.
[33] Csillik, B., Joò, F., Kasa, P., Yomity, I. and Kalman, Gy. (1964) Acta Biol. Hung. 15, 11–17.
[34] Brown, W. J. and Palay, S. L. (1972) Z. Anat. Entwickl. Gesch. 137, 317–334.
[35] Vijayan, V. K. and Wilson, D. B. (1975) Neurobiology 5, 228–234.