Histochemical Remarks on Monoamine Oxidase and Acetylcholinesterase in Some Regions of Amphibian Brain

A. CONTESTABILE and I. MUSSONI

Istituto di Anatomia Comparata dell'Università, Via Belmeloro 8, I–40126 Bologna (Italy), 1 April 1975.

Summary. The histochemical localization of monoamine oxidase and acetylcholinesterase has been studied in the cerebellum, optic tectum and telencephalon of three amphibian species. For acetylcholinesterase in particular, different histochemical methods and selective inhibitors have been used in order to investigate the actual nature of enzymatic activity revealed.

Many enzymatic patterns are well known in the brain of higher vertebrates by means of histochemical and biochemical methods[1]. Few data are, however, available on enzyme localization in the brain of lower vertebrates. Some results obtained in amphibians, in particular those concerning AChE localization, are sometimes based on procedures no longer used in histochemical practice[2], or are characterized by the non-use of selective inhibitors[2], or by unusual interpretation of inhibitor action[3,4]. For these reasons we have studied the histochemical localization of monoamine oxidase (MAO) and acetylcholinesterase (AChE) in some selected regions (cerebellum, optic tectum and telencephalon) of both the urodele and anuran brain.

Material and methods. For present work, we have used specimens of *Triturus cristatus*, *Bufo bufo* and *Rana esculenta*, sacrificed in April-June and August-September periods.

For AChE demonstration the brains were fixed for 2–4 h in 10% formol saline; cryostat sections, cut in transverse and sagittal planes, were incubated for 90 min at 20–22 °C in the media of GEREBTZOFF[5] and KARNOVSKY and ROOTS[6], containing alternatively acetylthiocholine or butyrylthiocholine iodide as substrate. The following inhibitors were routinely used: eserine 1 to 3×10^{-5} *M* to inhibit all cholinesterases, iso-OMPA 3×10^{-5} to 1×10^{-4} *M* and DFP 1 to 3×10^{-7} *M* to inhibit pseudocholinesterases[7–10].

For MAO demonstration the unfixed brains were immediately frozen and cut in the cryostat; the sections were incubated for 40 min at 35 °C in the medium of GLENNER et al.[11]. Some control sections were treated with the selective MAO inhibitor, nialamide, at 5×10^{-5} or 1×10^{-4} *M* concentration.

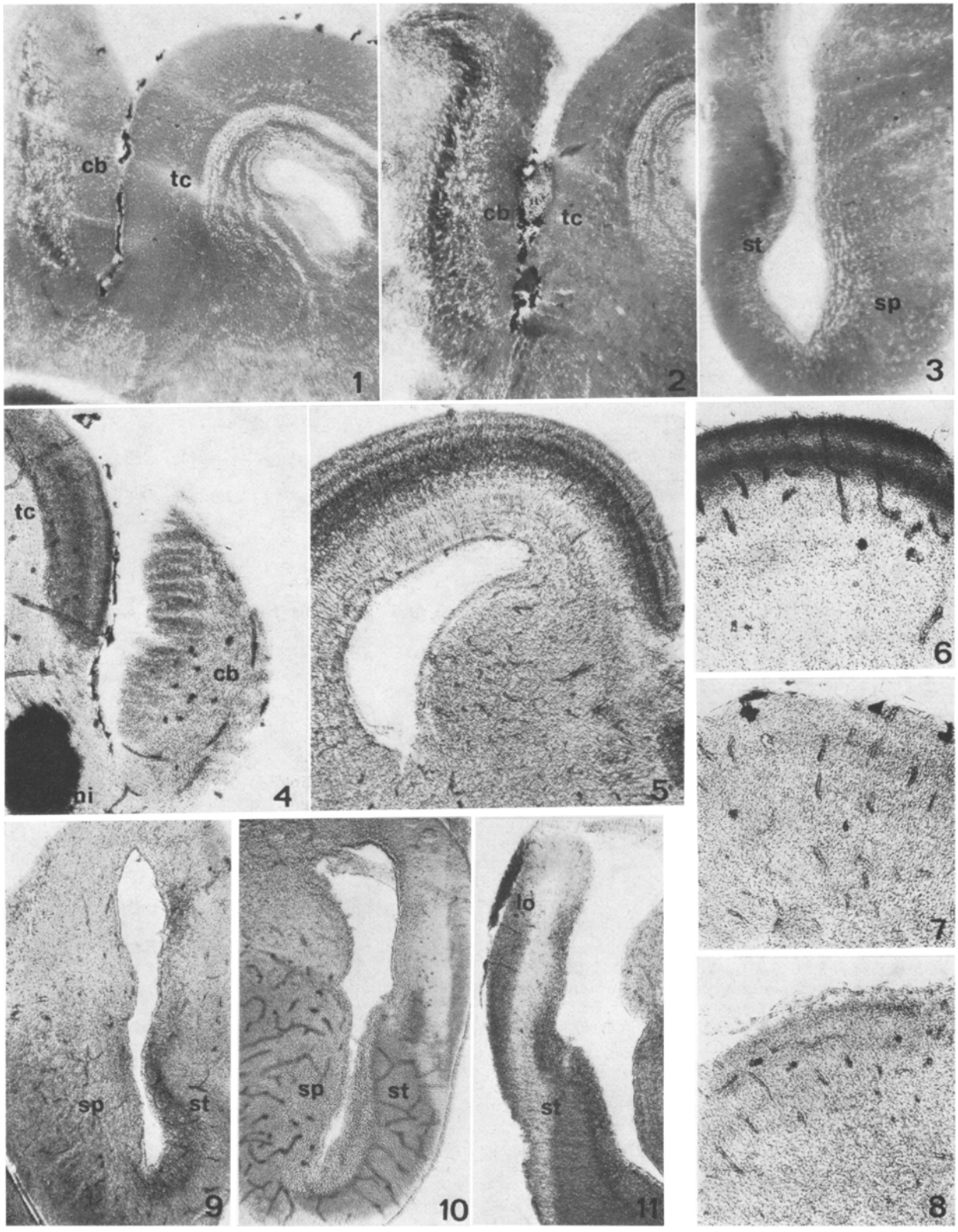
Results. No remarkable MAO activity is revealed by histochemical method in the brain of *Triturus*. In the anuran cerebellum, marked reaction is present in the fibrous layer and weaker reaction in the molecular one (Figures 1 and 2). The optic tectum of *Rana* and *Bufo* exhibits weak reaction in periventricular plexiform layers and more intense reaction in inner fibrous layer, while the reaction is of medium intensity in superficial tectal area (Figures 1 and 2). In the telencephalon the reaction is almost uniformly spread; MAO activity is a little stronger in septal and striatal areas (Figure 3). In any case, nialamide treatment causes almost complete disappearance of the histochemical reaction.

The atrophic cerebellum of *Triturus* does not show positive reaction for AChE, while in anuran cerebellum weak reaction is present in the granular layer and stronger reaction in the molecular one (Figure 4). In the optic tectum of *Triturus*, 2 bands of strong AChE activity are revealed in superficial layers, i.e. the afferent and the neuropilar layers (Figure 6). AChE localization is similar in the optic tectum of *Bufo* and *Rana* (Figures 4 and 5): in outer fibrous and plexiform layers the reaction is distributed in 3 bands; the deepest band shows stronger reaction and corresponds to *laminae* C (partly), D and E of POTTER's classification[12]; the underlying layer of pyramidal neurons shows weak reaction, while no reaction is present in all the other deep layers. The telencephalon of *Triturus* does not show positive reaction. In the anuran telencephalon a definite AChE activity is present in subpallial regions: the histochemical reaction is scattered in septal and striatal areas (Figures 9 and 10); moreover, in *Rana* a distinct reaction appears in a superficial band corresponding to the outer margin of epistriatum and lateral pallium, where the lateral olfactory tract runs. The other pallial areas do not show appreciable reaction.

In many areas of amphibian brain, one can notice a sharp reaction for AChE on capillary walls. This kind of localization is more evident using the GEREBTZOFF method and prevails in areas with high AChE activity. Butyrylcholinesterase (BuChE) activity is hardly visible in anuran brain, while in *Triturus* weak but definite reaction is present on capillary walls (Figure 7). Eserine treatment completely inhibits the histochemical reaction for AChE, except for weak reaction which persists on capillary walls of *Triturus*. (Figure 8). Finally no significant differences in the localization and intensity of the histochemical reaction have been observed in sections treated or untreated with iso-OMPA and DFP.

Discussion. MAO localization observed in anuran nervous centres partly corresponds to that described by KUSUNOKI et al.[13]. The main difference is the positive reaction in the fibrous layer of cerebellum; this kind of localization is a common feature also in other vertebrates[14–16]. Regarding the anuran telencephalon, our results are in agreement with those of NORTHCUTT[17].

[1] R. L. FRIEDE, *Topographic Brain Chemistry* (Academic Press, London 1966).
[2] S. C. SHEN, P. GREENFIELD and E. J. BOELL, J. comp. Neurol. *102*, 717 (1955).
[3] G. MINELLI and A. QUAGLIA, Riv. Biol. *61*, 63 (1968).
[4] G. MINELLI and A. QUAGLIA, Riv. Biol. *62*, 255 (1969).
[5] M. A. GEREBTZOFF, Acta anat. *19*, 366 (1953).
[6] M. J. KARNOVSKY and L. ROOTS, J. Histochem. Cytochem. *12*, 219 (1964).
[7] B. J. BAYLISS and A. TODRICK, Biochem. J. *62*, 62 (1956).
[8] M. A. GEREBTZOFF, *Cholinesterases* (Pergamon Press, London 1959).
[9] M. BRZIN, V. M. TENNYSON and P. E. DUFFY, J. Cell Biol. *31*, 215 (1966).
[10] A. G. E. PEARSE, *Histochemistry. Theoretical and Applied* (Churchill Livingstone, London 1972), vol. 2.
[11] G. G. GLENNER, H. J. BURTNER and G. W. BROWN, J. Histochem. Cytochem. *5*, 591 (1957).
[12] H. D. POTTER, J. comp. Neurol. *136*, 203 (1969).
[13] T. KUSUNOKI, H. ISHIBASHI and H. MASAI, J. Hirnforsch. *9*, 63 (1967).
[14] T. KUSUNOKI and H. MASAI, Archvm. histol. jap. *27*, 363 (1966).
[15] S. L. MANOCHA and G. H. BOURNE, J. Neurochem. *15*, 1033 (1968).
[16] G. MINELLI, Riv. Biol. *63*, 61 (1970).
[17] R. G. NORTHCUTT, J. comp. Neurol. *157*, 379 (1974).



Figs. 1 and 2. MAO activity in cerebellum (cb) and optic tectum (tc) of *Bufo* (1) and *Rana* (2). × 55. Fig. 3. MAO activity in the telencephalon of *Bufo:* sp, septal area; st, striatal area. × 40. Fig. 4. AChE activity in cerebellum (cb) and optic tectum (tc) of *Bufo*: ni, nucleus isthmi. GEREBTZOFF method; section treated with iso-OMPA 5×10^{-5} *M*. × 50. Fig. 5. AChE activity in optic tectum of *Rana*. KARNOVSKY and ROOTS method; section treated with iso-OMPA 5×10^{-5} *M*. × 40. Fig. 6. AChE activity in superficial layers of the optic tectum of *Triturus*. GEREBTZOFF method; section treated with DFP 3×10^{-7} *M*. × 55. Fig. 7. BuChE activity on capillary walls in the optic tectum of *Triturus*. KARNOVSKY and ROOTS method. × 40. Fig. 8. Eserine-resisting, non-specific esterase activity on capillary walls in the optic tectum of *Triturus*. KARNOVSKY and ROOTS method; section treated with eserine 3×10^{-5} *M*. × 40. Figs. 9–11. AChE activity in the telencephalon of *Bufo* (9) and *Rana* (10 and 11): lo, lateral olfactory tract; sp, septal area; st, striatal area. 9 and 11: KARNOVSKY and ROOTS method; 10: GEREBTZOFF method. Sections treated with iso-OMPA 5×10^{-5} *M*. × 40.

AChE activity in amphibian optic tectum appears prominent in superficial layers, where sensitive (mainly retinic) afferents discharge. In comparison with previous observations[2], we noticed a more differentiated AChE distribution in anuran optic tectum. This kind of distribution corresponds to different bands of retinic terminals revealed with different experimental methods[12,18,19]. AChE rich bands in superficial tectal layers might correspond to sites of action of the cholinergic inhibitory system described by STEVENS[20]. In the anuran telencephalon, AChE activity is prominent in sub-pallial regions, in agreement with observations made with a histochemical semi-quantitative method[17]. A characteristic difference in AChE localization between *Rana* and *Bufo* is the positive reaction corresponding to the lateral olfactory tract, observed only in the telencephalon of *Rana*.

Regarding AChE reaction on capillary walls, the use of two different histochemical methods, two different substrates and selective inhibitors permits us to draw some conclusions. In anurans the complete absence of histochemical reaction achieved by either eserine inhibition or the use of butyrylthiocholine as substrate, permits exclusion of interferences of non-specific esterases or pseudocholinesterases. This finding is confirmed by the lack of any remarkable inhibitory effect using the selective pseudocholinesterase inhibitors, iso-OMPA and DFP. Thus, from histochemical controls, it clearly results that the histochemical reaction is only due to true AChE. However, some of our observations suggest that AChE localization on capillary walls may be actually a false localization. In fact, this kind of localization is more or less evident using different histochemical methods. Furthermore, in nervous areas devoid of AChE activity, as for example the telencephalon of *Triturus* and some pallial regions of anurans, one cannot find any positive reaction on the capillary walls; this last finding might indicate a diffusion process from areas rich in enzyme activity and subsequent absorption of the enzyme on capillary walls. In *Triturus* the situation is little different, and it appears that also pseudocholinesterases and non-specific esterases may partly contribute to reaction on capillary walls; however histochemical controls do not confirm that this kind of reaction is only or prevailingly due to non-specific esterases in *Triturus*[3,4].

[18] G. SZEKELY, G. SETALO and GY. LAZAR, J. Hirnforsch. *14*, 189 (1973).
[19] S. H. CHUNG, T. V. P. BLISS and M. J. KEATING, Proc. R. Soc. London *187*, 421 (1974).
[20] R. J. STEVENS, Brain Res. *49*, 309 (1973).

Incorporation of Tritiated Uridine During Pachytene and Diplotene Stages in the Oocytes of the Japanese Quail (*Coturnix coturnix japonica*)

M. HARTUNG and A. STAHL

Laboratoire d'Histologie et Embryologie II, Faculté de Médecine, 27, boulevard J. Moulin F-13385 Marseille Cedex 4 (France), 11 July 1975.

Summary. Incorporation of ^{3}H-uridine was studied during pachytene and diplotene stages of quail oocytes. No labelling could be detected during early pachytene. During advanced and late pachytene, labelling simultaneously appeared on the macrochromosomes and on certain michromosomes in the zone where they emerge from the chromocentric surface periphery. The latter localization corresponds to the region of ribosomal RNA synthesis. At diplotene the same localizations were labelled with a considerably increased intensity.

Incorporation of RNA precursors during pachytene and diplotene in the oocyte has been studied by autoradiography only in Amphibians[1,2] and Invertebrates[3-6].

In a previous study, we have shown that the heterochromatic regions of quail microchromosomes fuse during pachytene to constitute the chromocenters. Euchromatic regions of the microchromosomes remain free, radiating around each chromocenter. During diplotene, nucleoli appear and develop in contact with the chromocenters and in strict relation with euchromatic segments of certain microchromosomes. These segments either encircle or penetrate the nucleolus[7], Since labelled nucleosides are incorporated early by precursors of ribosomal RNA, administration of tritiated uridine may contribute to the identification of the sites of nucleolar organization[8].

Material and methods. Cortex from the ovaries of hatching quails aged 12 and 24 h were sectioned into fragments of about 1 mm. Fragments were incubated in a medium of 80% Hanks solution and 20% fetal calf serum containing 100 µCi of H^3-uridine/ml (specific activity 25 Ci/m*M*, CEA France).

Incubation times were 5, 15, 20, 30, 45 and 60 min. Incubation was followed by a 5 min chase using the same medium containing non-radioactive uridine at a concentration of 1 mg/ml. Specimens were immediately fixed in methanol acetic acid (3/1) and treated by the method of LUCIANI et al.[9] which permits one to obtain isolated and well-spread germinal cells. Preparations were stained by acetic orcein and covered by Ilford K2 emulsion. Exposure times were 3 weeks, 1 month and 45 days.

Observations. (A) *Pachytene stage.* During early pachytene, prior to fusion of the heterochromatic zones of the microchromosomes, no radioactivity can be detected. Incorporation of tritiated uridine can only be detected in

[1] J. G. GALL and H. G. CALLAN, Proc. natn. Acad. Sci., USA *48*, 562 (1962).
[2] U. SCHEER, M. F. TRENDELENBURG and W. W. FRANKE, J. Cell Biol. *65*, 163 (1975).
[3] C. FAVARD-SERENO and M. DURAND, Devel. Biol. *6*, 184 (1963).
[4] A. FICQ, Natn. Cancer Inst. Monograph *23*, 311 (1966).
[5] N. K. DAS and M. ALFERT, Natn. Cancer Inst. Monograph *23*, 337 (1966).
[6] A. LIMA DE FARIA, in *Handbook of Molecular Cytology* (Ed. A. LIMA DE FARIA, American Elsevier, New York 1969), p. 278.
[7] A. STAHL, J. M. LUCIANI, M. DEVICTOR, A. M. CAPODANO and M. HARTUNG, Expl Cell Res. *91*, 365 (1975).
[8] N. GRANBOULAN and PH. GRANBOULAN, Expl Cell Res. *38*, 604 (1965).
[9] J. M. LUCIANI, M. DEVICTOR-VUILLET, R. Gagne and A. STAHL, J. Reprod. Fertil. *36*, 409 (1974).