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The polymorphism of acetylcholinesterase in different animal species and motor innervation

F. BACOU

with the technical assistance of Marylène ROSTAGNO

Station de Physiologie animale, INRA,
ENSA, place Viala, 34060 Montpellier Cedex, France.

Summary. Acetylcholinesterase in the muscles of birds and mammals has several molecular forms with the same catalytic activity ; these forms can be distinguished by their macromolecular properties. The presence of such asymmetric forms as A_{12} (triple tetramer with a collagen-like tail) is related to motor nerve functioning. Although, contrary to rat, they are always detected in nerve-free samples of chicken muscle, these asymmetric forms disappeared after various muscles of adult chicken and rabbit were denervated in our experiments. They appear in bird embryos at the time when the neuromuscular contacts are formed. The existence of asymmetric forms may depend on the contractile activity of muscle fibers and thus on the functioning of motor innervation. They could be good indicators of neuromuscular interactions.

Introduction.

Study of the installation of motor functioning during myogenesis is of the utmost importance in promoting the understanding of muscle development. It is not known if the trophic influence of the neuron, as shown in the adult (Buller *et al.*, 1960 ; Buller and Lewis, 1965), has the same importance during myoblast differentiation ; the reciprocal nerve-muscle interactions during this period are not well known either. The separation of the different molecular forms of acetylcholinesterase (AchE, E.C. 3.1.1.7) (see review by Rosenberry, 1975) seem to be a means of showing the existence of neuromuscular relations. AchE catalyses the hydrolysis of acetylcholine released at the cholinergic synapses in the nervous system or at the neuromuscular junction (Katz, 1969). In muscles, AchE is highly concentrated at the motor endplate where it terminates the action of the neurotransmitter on the post-synaptic membrane ; it is readily detected at the myotendinous junctions, in the sarcotubular system and also occurs in muscular areas which lack endplates (Tennyson *et al.*, 1977). Its physiological role in these regions is unknown. AchE occurs in vertebrate tissues in a number of molecular forms, as shown by their sedimentation coefficients on sucrose gradients (Massoulie and Rieger, 1969). Two types of molecular form may be

distinguished, a globular form (mono, di and tetrameric forms) and an asymmetric one containing one, two or three tetramers with a collagen-like tail (Massoulie *et al.,* 1980 ; Rosenberry and Richardson, 1977 ; Allemand *et al.,* 1981). None of these forms is specific to a particular function, except for the highest molecular weight asymmetric forms, A_{12}, which seems to be associated with motor endplates and/or functional activity (Hall, 1973 ; Jedrzejczyk *et al.,* 1981).

In this paper, we shall review studies showing the relationships between motor innervation and asymmetric forms. Our own work has been conducted along two main lines : (i) a study during the course of myogenesis in the chick embryo, carried out as soon as the formation of neuromuscular contacts was observed, and (ii) a study of denervation in adult chicken and rabbit to verify the permanent influence of the motor nerves.

Material and methods.

Fertilized eggs were obtained from our New Hamsphire breed. On day 20 of incubation, we dissected the *m. tibialis posterior* and on day 12 the inner part of the leg muscles ; on day 6 the hind limb buds were carefully dissected under a binocular lens, pooled and used in the experiments.

Muscle denervation.

a) *Chickens.* Six-month old New Hampshire hens were anaesthetized with ether. The *m. latissimus dorsi posterior* (m. LDP) was denervated by ligature and section of the common LDA-LDP nerve trunk. The denervated muscles were analyzed 4 weeks after denervation.

b) *Rabbits.* Five-month old female New Zealand white rabbits were anaesthetized with nembutal, and the semimembranosus branch of the sciatic nerve of one leg was ligatured and sectioned. The other leg served as a control, but rabbits in which neither leg had been denervated were also used. The *m. semimembranosus* was dissected (the red inner homogeneous part was discarded) and analysed 4 to 5 weeks after denervation.

Extraction procedure and sedimentation analysis. — Muscle samples were homogenized in glass homogenizers in 10 volumes of 0,01 M Tris HCl buffer, pH 7.2, containing 1 M NaCl, 1 p. 100 Triton X 100 and 1 mM EGTA. The homogenates were centrifuged for 20 min at 35 000 × g prior to use. Usually, 150 to 200 µl of the supernatant were layered on top of 10 to 25 p. 100 linear sucrose gradients made up with extraction buffer and centrifuged for 24 h on a MSE 3 × 25 ml rotor at 100 000 × g at 4 °C. Sedimentation constants for the various forms of AchE were calculated by comparison with those of horse liver alcohol dehydrogenase (ADH : 4.8 S) and β-galactosidase (βG : 16 S) added to the samples prior to sedimentation.

Enzyme assays. — AchE was detected in each of the thirty-five or so fractions from each gradient by the method of Ellman *et al.* (1961) using 0.1 mM

iso. OMPA (tetraisopropyl-pyrophosphoramide) in chicken and 0.1 mM ethopropazine in rabbit experiments. After preincubation at 37 °C for 20 min in 1.5 ml of this medium to permit full inactivation of butylrylcholinesterase (BchE, E.C. 3.1.1.8) by ethopropazine or iso. OMPA (Austin and Berry, 1953), the reaction was initiated by the addition of 0.75 mM acetylthiocholine iodide and 0.5 mM dithiobisnitrobenzoic acid (DTNB). Incubations were carried out at 35 °C for 50 min.

Reagents. — All the biochemical reagents used in this study were of analytical grade. Acetylthiocholine iodide, DTNB, ADH, βG were obtained from Boehringer Mannheim Co ; iso. OMPA, EGTA and Triton X 100 were purchased from Sigma Chemical Co ; ethopropazine was a gift of Opodex Co.

Results.

Molecular forms of AchE in chick embryo muscles of different ages (fig. 1). — The sedimentation profile of AchE extracted from hind limb muscles of chick embryos is shown in figure 1 (A, B, C). On day 6 of incubation, the hind limb bud was about 3 mm long ; it is difficult to dissect muscles free of the skin and bone, so the entire limb was analyzed for AchE. The enzyme was essentially represented by 6.8 S (35 p. 100) and 11.2 S (62 p. 100) forms. The 19.2 S form (2 p. 100) appeared (a 22.6 S form — 1 p. 100 — was present at this stage and disappeared later). On day 12, the relative proportions of the three principal forms represented 28, 42 and 30 p. 100, respectively, of AchE activity. On day 20, a 14.5 S form (16 p. 100) appeared but the 19.2 form was then preponderant (60 p. 100), while the 6.8 S and 11.2 S forms corresponded to 5 and 19 p. 100 of the enzyme activity.

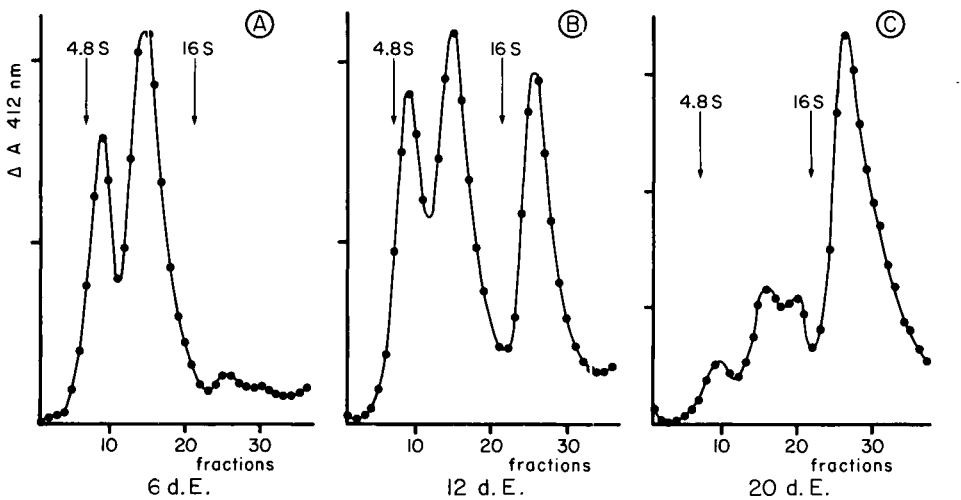

FIG. 1. — *Changes in acetylcholinesterase molecular forms during embryonic development of chick leg muscle.* A) 6-day old embryo : limb bud ; B) 12-day old embryo : inner leg muscles ; C) 20-day old embryo : *m. tibialis posterior.*

AchE in normal and denervated chicken LDP muscle (fig. 2 A). — The specific activity for the AchE was very weak in control m.LDP (0.5 ± 0.05 mU/mg protein* in our experiments). Three forms, 6.1 S (25 p. 100), 13 S (18 p. 100) and 19.3 S (6 p. 100), appeared. After 4 weeks of denervation, specific AchE activity increased by 30-fold (15.6 ± 4.9 mU/mg protein [1] in operated muscles). The enzyme was essentially constituted of the 6.1 S form with a shouldering of the 13 S form.

AchE in normal and denervated rabbit m. semimembranosus (fig. 2 B). — Four principal forms — 5.2 S (57 p. 100), 12.5 S (7 p. 100), 16 S (25 p. 100) and 19 S (11 p. 100) — have been shown in the control. Four to 5 weeks after denervation, specific AchE activity increased by 15-fold from 2.7 ± 0.9 mU/mg protein* in the controls to 42.8 ± 9.7 mU/mg protein [1] in operated muscles. As in the chicken, one light form, 3.6 S, was mainly revealed with a shouldering at 12.8 S. The 16 S form had disappeared, while the 19 S form represented only 1 p. 100 of the total activity.

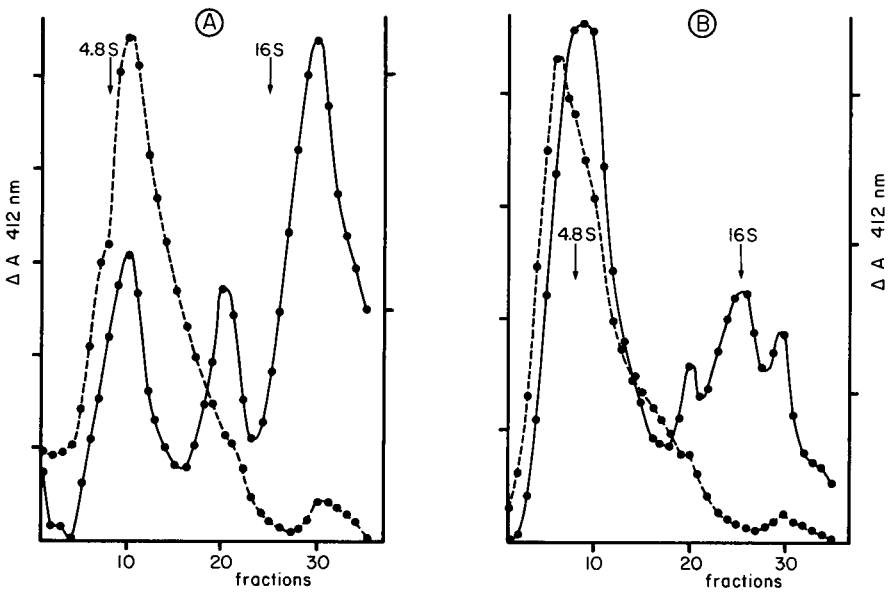

FIG. 2. — *Molecular forms of acetylcholinesterase in control and denervated chicken or rabbit muscle.* A) control and denervated chicken m. LDP ; B) control and denervated rabbit *m. semimembranosus.* ●——● control muscles, ●------● denervated muscles.

Discussion.

The alteration of the sedimentation velocity profile of AchE in 6 to 20-day old chick embryos was essentially characterized by a decrease of 6.8 + 11.2 S light forms and by an increase in the 19.5 S form. After 6 days of incubation, the nerve

[1] Mean of 3 to 5 experiments ± SE.

pattern of the leg had approximately acquired its definitive configuration (Fouvet, 1973) but muscle cell innervation was just commencing. The way the nerve-muscle contacts are established and their electrical and trophic properties are not yet well known. The establishment of nerve-muscle connections seems to be the starting point of coordinated molecular events, characterized, in our observations, by the appearance of the 19.5 S form. Is this form « endplate specific » (Koenig and Koenig, 1977 ; Koenig and Vigny, 1978 a ; Sketelj and Brzin, 1980), or is it induced in muscle tissues by the nerve (Fernandez *et al.,* 1979 a ; Weinberg and Hall, 1979) ? These questions have been the subject of numerous papers these last few years (Massoulie, 1980 ; Koenig, 1979) and there is now a consensus of opinion as to the existence of linked species differences.

In every case in the rat, the A_{12} form is localized where the fibers have neuromuscular contacts, *i.e.* endplate zones or when the neuromuscular contacts appear during myogenesis (Rotundo and Fambrough, 1979). Nerve-free segments or denervated muscles are deprived of the 16 S form (Fernandez *et al.,* 1979 b). Thus, in the rat, the high molecular weight of the 16 S form may be considered as a biochemical indicator of nerve-muscle contacts (Vigny *et al.,* 1976).

In human muscles and in twitch muscles of the chicken, the A_{12} form is not restricted to the endplate zone ; it is present in nerve-free segments in the same proportions as in the neuromotor area (Silman *et al.,* 1978). However, this form is absent in non-innervated muscles. The link, heavy form A_{12}-motor innervation, is always true. But the enzyme is not localized only at the endplate level and the motor nerve has solely an inductive role ; there is still inductive unity but no localization unity. Thus, in humans as in chickens, the A_{12} form cannot be considered as a biochemical indicator of the motor endplate. However, its presence in only innervated muscle confirms its relations with the neuromotor function. This aspect constitutes the point of convergence of the indicator properties of the A_{12} form in the different studied species and raises the question of the nature of inductive nervous factors (Sohal and Holt, 1980).

This problem is usually a subject of controversy. It seems, however, that motor activity plays a preponderant role in the presence and maintainance of the A_{12} form. Since the work of Lomo and Rosenthal (1972), the influence of motor activity on muscle properties has been studied often both *in vivo* and *in vitro. In vitro* studies have shown the difference between the roles of neurotrophic and neuromotor activity in the induction and maintainance of the A_{12} form. Our personal observations on 21-day old cultures of chicken pectoralis major (Nougues and Bacou, to be published), as those of Kato *et al.* (1980), show that the continued contractions of fibers grown *in vitro* are sufficient to determine the existence of the A_{12} form ; nerve-muscle contact is not necessary (Rubin *et al.,* 1980). Work in the rat is more advanced. It has been shown that the A_{12} form is synthesized *in vitro* in ordinary muscle cultures only if the cultured myoblasts have been taken from tissues previously containing this form *in vivo* (Koenig and Vigny, 1978 b). In other words, the neuron is needed to induce the synthesis of this A_{12}-specific protein in rat muscle tissue which keeps this information and translates it at a biochemical level, providing that motor activity continues. This has been shown by denervated

muscles, deprived of motor activity, in which the heavy form disappears. Motor activity would be the means by which muscle tissue expresses the properties and potentialities transmitted to it by the neuron. Once acquired, these properties remain myogenic (Bacou and Nougues, 1980), and the nerve affects the muscle only by its motor function. The nerve may thus have two complementary but distinct influences on muscle : trophic influence during myogenesis and motor influence during postnatal life, that is when the structures necessary to muscular functioning have been established.

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Résumé. Dans les muscles des oiseaux et des mammifères, l'acétylcholinesterase présente plusieurs formes moléculaires de même activité catalytique, distinctes par certaines de leurs propriétés macromoléculaires. La présence de formes asymétriques et en particulier de la forme A_{12} (triple tétramère à queue de structure collagénique) est liée à la fonction nerveuse motrice. Bien que toujours détectées dans les extraits de régions aneurales de muscle de poulet, contrairement à ce que l'on observe chez le Rat, elles disparaissent après dénervation de différents muscles, chez le Poulet et le Lapin adultes dans nos expériences. Au cours de l'embryogenèse de l'oiseau, elles apparaissent au stade où se forment les contacts neuromusculaires. L'existence des formes asymétriques pourrait dépendre de l'activité contractile des fibres musculaires, donc d'une innervation motrice fonctionnelle. Par ce biais, elles constitueraient un marqueur intéressant des interactions neuromusculaires.

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