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Molecular forms of acetylcholinesterase and pseudocholinesterase in chicken skeletal muscles : their distribution and change with muscular dystrophy

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Summary. Chicken muscles offer several significant advantages for the use of cholinesterase as a marker of nerve-muscle interactions. A series of molecular forms of chicken muscle acetylcholinesterase (AChE), and likewise of pseudocholinesterase (ψChE), has been defined. The form of AChE inside the endplates of fast-twitch muscle is H_{2c} (20 S), with a collagenous tail. The same is true for ψChE. The changes in these forms in the muscle with embryonic development, with muscle fibre-type composition and under the influence of inherited muscular dystrophy. are described quantitatively.

Introduction.

Molecular markers are needed to study the influence of neurones on muscle cells, and acetylcholinesterase (AChE), due to its concentration at the motor endplates, has for some time attracted attention as a possible protein marker of this type (for a review, see Rubin *et al.*, 1980). When rat muscle, for example, is chronically denervated *in vivo,* AChE decreases both in total content and in the form (16 S) suggested to be endplate-specific (Hall, 1973 ; Vigny, Koenig and Rieger, 1976 ; Sketelj and Brzin, 1980). While most studies of AChE as such a marker have been made on mammalian muscles, chicken muscles offer a number of advantages for a detailed pursuit of this enzymic probe :

(i) the chicken has a number of useful muscles that are of a virtually pure fibre type, whereas mammalian muscles generally have very mixed fibre types, leading, as we shall show, to complexity in that system ;

(ii) the stages of innervation and muscular development are much more accessible for study in the chick egg than in the mammalian embryo *in utero* ;

(iii) a mutant fowl, the dystrophic chicken, is available which has a major de-regulation of AChE biosynthesis in the muscles (Wilson, Montgomery and

Asmundson, 1968 ; Lyles, Silman and Barnard, 1979) but not in the motor nerves (Di Giamberardino, Couraud and Barnard, 1979) ;

(iv) the embryonic chick muscle and some adult chicken muscles contain significant amounts of pseudocholinesterase (ψChE), the enzyme related to AChE but of different substrate and inhibitory specificity. Because the amounts of ψChE are very high in dystrophic chicken muscles, it can serve as a second marker for studying neural influences (Silman *et al.,* 1979).

This paper summarizes some of our findings on chicken muscle AChE and ψChE relating to the above questions.

Forms of AChE in chicken muscles. — As with rat muscle (Hall, 1973), the AChE of chicken muscles is completely extracted into solution by Triton X-100/1M NaCl (Silman, Lyles and Barnard, 1978). Multiple molecular forms, present in the extract, are conveniently distinguished by their sedimentation coefficients in sucrose density gradients in the same medium (fig. 1). We classify the types of forms that occur into three broad size groups : light (L), medium (M) and heavy (H) (table 1). The H forms (about 20 S and 15 S), with properties

TABLE 1

Sedimentation coefficients of chicken muscle forms of cholinesterases[a]

Form :	L_1	L_2	M	M_c [b]	H_{1c}	H_{2c}
AChE	4.7 S	6.6 S	11.1 S		14.7 S	20.0 S
ψChE	4.0 S	5.9 S	10.5 S		14.1 S	19.2 S

[a] From Lyles, Silman and Barnard (1979) in their conditions. [b] M_c has not been found so far as a native species in chicken muscles, although it has been found in extracts of mammalian muscle (Bon, Vigny and Massoulié, 1979).

comparable to the 18 S and 14 S species of the AChE of the electric organ of *Electrophorus* (Anglister and Silman, 1978 and references cited therein), have been well characterized chemically. These hydrodynamically asymmetric species have a remarkable molecular structure, containing 12 or 8 catalytic subunits (of ~ 80,000 molecular weight each), respectively, in a head unit, combined in groups of 4, and all linked to a collagenous tail about 500 Å long (Anglister and Silman, 1978 ; Bon and Massoulié, 1978). An equivalent form of about 16 S, which is similarly asymmetric and modifiable by collagenase, occurs in mammals (Bon, Vigny and Massoulié, 1979) along with lighter globular forms. In fast-twitch muscle of mature chickens the major form is about 20 S in size and its extraction requires both non-ionic detergent and 1M salt (Silman, Lyles and Barnard, 1978). It, likewise, is non-globular and aggregates in low-salt media (Bon, Vigny and Massoulié, 1979 ; Allemand *et al.,* 1982). On treatment with collagenase, we find (Lyles *et al.,* 1982) that a transformation occurs similar to that found with eel 18 S AChE, in which, due to the cleavage of the collagen tail, a 21 S and a 16 S species replace the 20 S form, initially producing a faster-sedimenting, more

symmetric form and then a detachment of some subunits. Hence, the chicken muscle 20 S AChE is also a collagen-tailed structure ; this conclusion is supported for both the 20 S and the 15 S forms by the results of partial tryptic digestion (Allemand *et al.,* 1981). The other forms extracted from chicken muscle are unaffected by collagenase, do not aggregate in the absence of high salt concentration and behave as globular proteins. To define the two groups to which the various-sized forms belong, we have therefore distinguished by the subscript c (table 1), those forms for which there is evidence of a collagenous tail in the molecule. In mammalian muscle there is evidence for two different forms with 4 catalytic units, one collagenous and the other globular (Bon, Vigne and Massoulié, 1979), which we have termed M and M_c, but the latter is not normally seen in chicken muscles. The size of the catalytic subunit of chicken AChE has been deduced to be larger (about 120,000 daltons) than that of mammalian AChE (Allemand *et al.,* 1981), thus accounting for the higher sedimentation coefficients of the corresponding forms. However, this size was only approximately estimated from hydrodynamic data and, in general, the size, nature and stoichiometry of all the non-collagen subunits has not yet been clearly established in either mammalian or avian muscle AChE. Since recent data shows that non-catalytic, non-collagen subunits may be present, even in electric organ AChE (Lee and Taylor, 1980), the picture of the muscle H_{2c} form as a dodecamer of identical subunits, linked to a collagen tail, may be a simplification, and we prefer to use the designations of table 1 rather than assign subunit numbers to each form.

An important requirement in comparing the molecular species of muscle AChE in different cases is to avoid proteolytic modification which, due to the variety of muscle proteases and their high levels, occurs easily, giving artefactal active forms. A set of protease inhibitors, present at the stages of homogenisation, extraction and analysis, is needed to preserve all the native forms intact (Silman, Lyles and Barnard, 1978). Among these inhibitors are ethylenediaminetetra-acetic acid (EDTA) and ethyleneglycol-bis (β-aminoethyl)-tetra-acetic acid (EGTA) to chelate the divalent metal activators of some proteases ; it is interesting that Barat *et al.* (1980) recently found that EDTA is needed in the chicken or mammalian nervous system for extraction of the H_{2c} form, which can reach 15 p. 100 of the total if EDTA is used. This form is extracted from chicken muscle with or without these chelators, but, if a small percentage were bound similarly (presumably through Ca^{2+}), it would not be missed in the extraction which we use routinely.

Forms of ψChE in chicken muscles. — ψChE accompanies AChE in muscles. (In the chicken this enzyme does not have the same specificity as butyrylcholinesterase in mammals and, therefore, the former term is preferred.) ψChE exists in a series of forms parallel to those of AChE (Silman *et al.,* 1979 ; Lyles, Silman and Barnard, 1979) ; their sedimentation coefficients are always a little lower than for the corresponding AChE forms (table 1). The low-salt aggregation of the H forms of chicken ψChE is the same as for AChE (Allemand *et al.,* 1981), and we found the same change with collagenase (Lyles *et al.,* 1981) as seen with chicken and mammalian H AChE. The properties of all the ψChE forms (Allemand

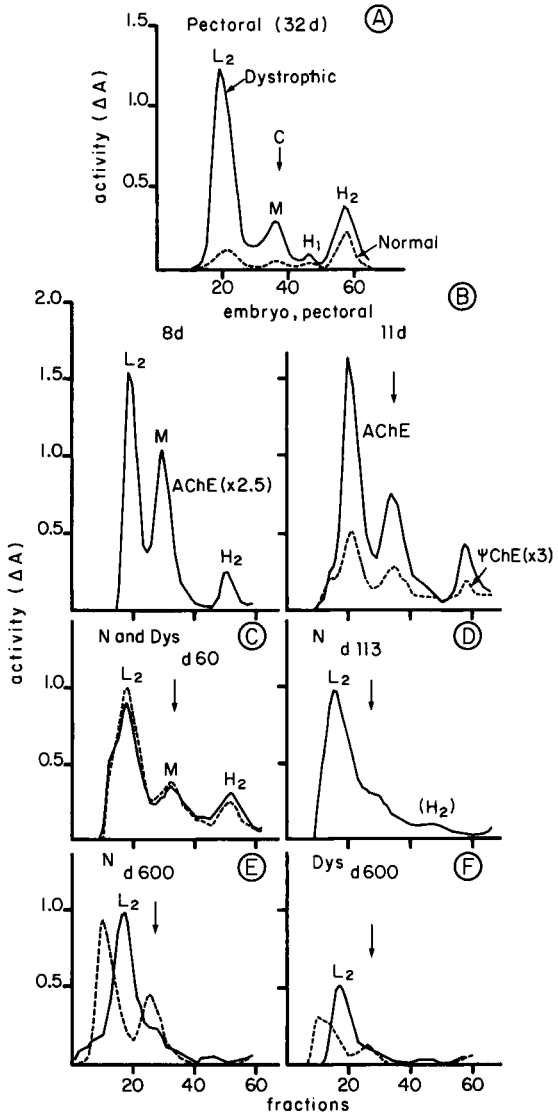

FIG. 1. — *Sucrose density gradient separations of the molecular forms of AChE or ψChE in chicken muscle extracts.* In all gradients, the heavier species are on the right ; the arrow indicates the catalase (11.4 S) marker.

A : Broken line : pectoral muscle from a 32-day old normal chicken. Note that by this age, the H_{2c} form (marked H_2 on these gradients) is predominant. Solid line : pectoral muscle from a chicken homozygous for muscular dystrophy of the same age, using the same muscle weight. Note the great elevation of L_2 and the significant elevations of M and H_{2c}.

B : Pectoral muscle from normal 8-day and 11-day old embryos. Solid lines, AChE : amount of muscle from the 8-day sample is 2.5 times that of the 11-day due to the lower concentration at that age. The H_{2c} peak appears before day 8 of incubation *in ovo*. Broken line : ψChE at 11 days (with 3 times the sample amount).

C : ALD muscle from normal (solid line) or dystrophic (broken line) chickens at 60 days. The same muscle weights were used for both (AChE only). Note that L_2 is the major form in the slow-tonic muscle and that there is no change with dystrophy.

D, E : Normal ALD at 113 days or 600 days. Solid line : AChE. Broken line in E : ψChE (4 times the amount of sample). Note the disappearance of H_{2c} of both enzymes.

F : ALD from a dystrophic bird at 600 days. Solid line : AChE. Broken line : ψChE (1.7 times the amount). Note the identity with the normal control in E. The native major form of ψChE is L_1.

et al., 1981 ; Lyles *et al.*, 1981) are sufficiently parallel to those of the AChE forms to permit us to use the corresponding designations of the former (table 1).

Material and methods.

For data not taken from publications elsewhere, the birds and experimental methods were as described by Lyles, Silman and Barnard (1979) and Lyles *et al.* (1981).

Results and discussion.

Molecular forms in fast-twitch muscles. — In the mature bird, H_{2c} accounts for the great majority of AChE activity (fig. 1) in muscles such as the pectoral and posterior latissimus dorsi (PLD). These muscles, of almost pure fibre type in the normal chicken, have > 99 p. 100 (pectoral) and 90-95 p. 100 (PLD) type-IIB (fast-twitch glycolytic) fibres, so this pattern seems characteristic of that fibre type. In contrast, ψChE exists mainly in L forms ; this difference is exemplified as early as 1 month after hatching (last line in each of tables 2 and 3). At older ages these trends are still more pronounced. However, the concentration of total ψChE in these muscles is very low, being only about 5-10 p. 100 of that of the total AChE at 2 months of age (Lyles *et al.*, 1981).

Changes of molecular forms with embryonic development. — In the embryonic pectoral muscle, L_2 is the major at day 14, but M and H_{2c} are prominent (fig. 1 B). H_{2c} is easily distinguishable by day 8 *in ovo* and increases progessively (table 2). By day 19, H_{2c} is the major species and H_{1c} is detectable. The overall AChE concentration in the muscle likewise increases with embryonic development, but subsequently declines sharply (table 2). Similar progression in the forms is found for the mixed fibre muscles of the leg and the wing. The fast-twitch PLD muscle at days 8-9 *in ovo* also shows the pectoral pattern.

TABLE 2

The changing forms of AChE in normal developing pectoral muscle

Day	Percentage of total activity in : L_2	H_2	Total activity (U/g)[a]
In ovo			
8	47	8	2.2
11	52	11	7.1
14	42	19	5.5
16	49	23	3.7
19	14	55	7.0
Ex ovo			
7	14	42	5.3
32	18	52	0.13

[a] Total AChE activity per gram wet weight of muscle.

Throughout embryonic development of the fast-twitch muscle, the major form of ψChE is M. H_{2c} becomes detectable at the same stage as H_{2c} AChE (table 3) and increases until it equals the amount of M at hatching. Thereafter, both the total amount of ψChE and the amount of H ψChE fall abruptly. At this time, the changes are probably associated with the transformation from slow-oxidative to fast-glycolytic fibres in those muscles.

TABLE 3

The changing forms of ψChE in normal developing pectotal muscle

	Day	Percentage of total activity in :	
		$L_1 + L_2$	H_{2c}
In ovo			
	8	41	4
	14	26	16
	16	11	27
	19	17	39
Ex ovo			
	32[a]	76	12

[a] The sedimentation profiles for ψChE in muscles after hatching (Lyles, Silman and Barnard, 1979) are distorted due to the contribution of M and L ψChE from the blood plasma present. The value given here is correct, being obtained from muscles freed of blood by saline perfusion *in situ*. This correction is not necessary for profiles of AChE (negligible in normal chicken blood) nor for dystrophic fast-twitch muscle (in which muscle ψChE is much greater).

Innervation of the spectral muscle starts at 7-8 days *in ovo* and continues gradually up to about 16d (Hirano, 1967 ; Ashmore, Kikuchi and Doerr, 1978). The findings with AChE and ψChE forms suggest that the assembly of the H_{2c} structure is consequent upon synapse formation *in vivo*.

Molecular forms in other muscle types. — The musculature of birds is noteworthy for the frequent occurrence of slow-tonic fibres (very rare in mammals), isolated in pure or almost pure tonic muscles. The anterior latissimus dorsi (ALD) muscle has generally been studied as the standard example of such a muscle. The contractile properties and pattern of neural impulse traffic to this type of muscle are entirely different from those of the fast-twitch muscles. It is interesting, therefore, to find that the patterns of the AChE and ψChE forms produced are also quite different in that muscle. At 2 months, the major form in the ALD is L_2, and H_{2c} is relatively small (fig. 1 C). When the bird is fully mature, the H forms virtually disappear (fig. 1 D, E). Cytochemical (Silver, 1963) and quantitative (Buckley and Heaton, 1971 ; Jedrzejczyk *et al.*, 1973) analyses show that the endplates of the mature ALD still contain a high concentration of AChE, which must be in the L_2 and/or M forms.

ψChE also shows a loss of H forms (fig. 1 C, E), but L_1 and M are still present. In this case, the pronounced differences from the patterns and time-

courses of fast-twitch muscle are consistent with a differential regulation of these enzymes by the various innervations, either directly or due to different impulse traffic patterns.

There are other avian slow-tonic muscles, but they have not been as well studied as the ALD. Can the ALD pattern be applied in general to all slow-tonic muscles ? The plantaris is, by fibre-typing analysis (Barnard, Lyles and Pizzey, 1981), another slow-tonic chicken muscle. However, it has a large amount of H_{2c} AChE as well as a major L_2 form (fig. 2). Fast-twitch fibres are almost absent from this muscle, at least to the level of < 5 p. 100. We do not know the reason for this difference in the AChE forms, but it is obvious that the regulation of AChE forms is sensitive to local innervation.

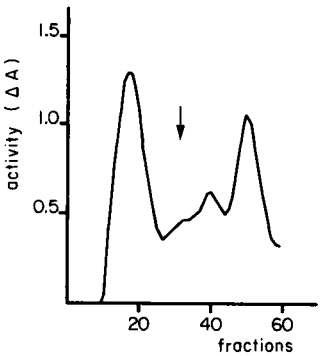

FIG. 2. — *Plantaris muscle (normal chicken). AChE forms.* L_2 is major, but H_{2c} is prominent. This muscle is, by electrophysiological analysis (Barnard, Lyles and Pizzey, 1982), slow-tonic and its fibres are multiply-innervated as in the ALD.

Localisation of the molecular forms. — For some time, the 16 S AChE form (H_{2c}) of mammalian muscle has been thought to be located at the motor endplates. Hall (1973) and Vigny, Koenig and Rieger (1976) showed that it was present in neural but absent in aneural muscle segments and disappeared from the former on denervation. However, such analysis after gross excision of endplate-rich zones does not exclude the possibility that some other forms are present, both inside and outside the endplate. Further, the specific association of H_{2c} with the endplates has been questioned after similar experiments on human (Carson *et al.*, 1979) and immature rat (Sketelj and Brzin, 1980) muscles. The lack of H_{2c} at the chicken ALD endplate (fig. 1) now shows clearly that this form is not of necessity at the endplate. Since the association with the endplate in fast-twitch muscles also might not hold, it was investigated separately.

This was accomplished by micro-dissecting individual endplates and analyzing them (Jedrzejczyk *et al.*, 1981). The results of this investigation showed unequivocally that the fast-twitch muscle endplate in mature chickens contains the H_{2c} form of AChE with a trace of H_{1c} (fig. 3 A). The significant amount of L_2 found in the whole muscle was not present there, being extra-junctional. The same was true for ψChE (fig. 3 B) : only H_{2c} was present, although >90 p. 100 of the ψChE in the same muscle was L_1 and M.

The tailed AChE molecule is presumed to be bound to the basal lamina at the endplate and the tailed ψChE may be similarly attached. Denervation of the ALD

muscle in 1-day chicks (in which considerable H_{2c} AChE and H_2 ψChE are still present) shows that *both* of these forms disappear within a few days (Silman *et al.*, 1979). The same is true of mature PLD muscle (Lyles *et al.*, 1982). These observations are consistent with the endplate location and with neural control of the assembly of the tailed form.

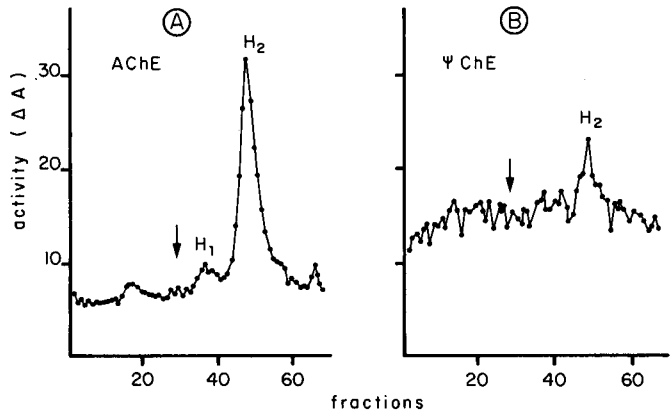

FIG. 3. — *Analysis of complete extracts of micro-dissected individual endplates* (30 for each sample). Activities measured with [^{3}H] acetylcholine (+ appropriate inhibitors). A : AChE. B : ψChE. (The background in a blank incubation was not subtracted in either case, and if done would reduce the base-line around the peaks to zero). (From Jedrzejczyk *et al.*, 1981).

Effects of muscular dystrophy on AChE and ψChE. — There are characteristic pathological changes in those muscles of chickens with homozygous muscular dystrophy where type-IIB fibres predominate, which become pronounced a few weeks after hatching (Wilson, Montgomery and Asmundson, 1968 ; Pizzey and Barnard, 1981). The tonic muscles do not show

TABLE 4

Increase in enzyme content (units/gram of muscle) in dystrophic muscles

Muscle	Predominant fibre type[b]	Age (days)	Dys/Normal	
			AChE	ψChE
Affected				
Pectoral	IIB	60	20.0	~ 50
PLD	IIB	60	5.7	15
Biceps[a]	IIB	56	6.4	
Unaffected				
ALD	Tonic	60	1.0	0.9
Sartorius (red)	I,II A	42	1.0	1.2

[a] Randall and Wilson (1980).
[b] The fibre typing of these mammalian muscles and its application to chicken muscles is described by Barnard, Lyles and Pizzey (1982).

these changes for many months. As first recognized by Wilson, Montgomery and Asmundson (1968), there is a remarkable increase in AChE and ψChE production in the affected muscles. This is best quantitated in the Triton/NaCl/EDTA complete extracts (Silman, Lyles and Barnard, 1978) described above, expressing the results as the enzyme ratio in dystrophic as compared to normal muscle (table 4). The type IIB fibres show a de-regulation of both AChE and ψChE synthesis which varies with the muscle. Both tonic muscles and those red muscles largely comprising oxidative twitch-fibres (types I and IIA) are essentially unaffected. This is illustrated for the ALD muscle in fig. 1 C, which shows that both the amount of AChE and the pattern of its forms is the same in dystrophic and normal birds.

In contrast, the ratios of the AChE forms are completely changed in the affected muscles. Figure 1A shows a great rise in L_2 in all cases, and a lesser but significant rise in H_{2c} (Lyles, Silman and Barnard, 1979). Micro-dissection studies have demonstrated an accumulation of excess H_{2c} and L_2 in the zone *around* the endplate (Jedrzejczyk *et al.*, 1981).

TABLE 5

Changes in AChE and ψChE forms during development of dystrophic (D) and normal (N) pectoral muscle[a]

		Age of birds (days)			
		19 *in ovo*	4	7	32
AChE, p. 100 L_2	(N)	14	21	14	18
	(D)	16	37	36	51
AChE, p. 100 H_{2c}	(N)	55	39	42	52
	(D)	53	28	28	21
ψChE, p. 100 L_1	(N)	13	16	9	6
	(D)	17	42	38	39
ψChE, total (mU/muscle)	(N)	222	143	239	< 300
	(D)	111	208	643	18,160

[a] All values are the means for 4 birds. The value given is the percentage of the total of AChE (or ψChE) present which is contributed by the molecular form specified, *or* the total ψChE content of the entire muscle.

TABLE 6

Changes in AChE during the development of dystrophic (D) and normal (N) flexor carpi ulnaris muscle[a]

		Age of birds (days)		
		in ovo		
		16	19	37
p. 100 L_2	(N)	40	39	7
	(D)	40	41	48[b]
p. 100 H_{2c}	(N)	21	30	77
	(D)	25	28	31[b]

[a] Details are for fig. 4 and table 5.
[b] Difference from normal significant at $P < 0.01$. Other differences not significant.

In the embryo, the ratios of the molecular forms of AChE (or ψChE) do not differ significantly between normal and dystrophic birds in any type of muscle tested (tables 5, 6). The first manifestation of a difference is in the total quantity of one or the other of the enzymes in fast-twitch muscles (pectoral and PLD) ; there is about 50 p. 100 less per muscle or per gram wet weight in the dystrophic muscle for one or two days before hatching (Lyles, Silman and Barnard, 1979). Table 5 shows ψChE levels in the pectoral muscle. After hatching, this trend reverses to give enormous increases in the dystrophic muscle. Also shown in table 5 are the contributions of the main L and H forms present before and after hatching ; distinct shifts, which are already significant at 4 days post-hatching, occur in the light forms of both AChE and ψChE in dystrophic muscle. Hence, the assembly of the AChE or ψChE molecule is affected at a very early stage in avian dystrophic development. As noted, dystrophy has some effects on these enzymes even *in ovo,* at the stage when the fibre types begin to differentiate. This precedes detectable histopathological changes in the muscle fibres and suggests that pathogenetic aberrations do occur during embryonic development.

Histopathological changes occur more slowly in dystrophic chicken muscles which are more distal anatomically and of mixed fibre type, but still have a majority of type-IIB fibres. An example is the flexor carpi ulnaris muscle in the lower wing, which is affected only slowly as the bird matures. This type of muscle is therefore mid-way between the two groups of muscle distinguished in table 4. At 5 weeks of age it still has not shown a rise in AChE (fig. 4). Nevertheless, the depression

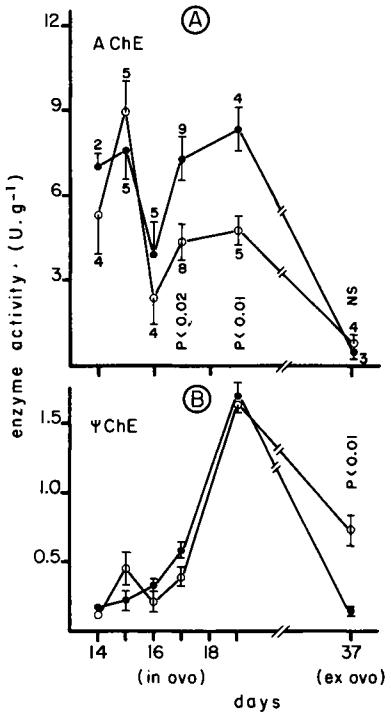

FIG. 4. — *Changes in the concentrations of* (A) *AChE and* (B) ψ*ChE in the developing FCU muscle in normal and dystrophic chickens.* Breaks in the lines represent the day of hatching. Each point is the mean of the number (n) of birds assayed as indicated above the bars, which represent S.E.M. ; n for each age is identical in A and B. The degree of significance of the difference between normal and dystrophic enzyme levels in indicated, where appropriate, by P as determined by t-test. Normal (●——●) ; Dystrophic (○——○).

of AChE production in the embryo occurs in the same way under the influence of the dystrophic gene ; likewise, the assembly of H_{2c} AChE is depressed and L_2 accumulates, as in the rapidly-affected muscles, despite the absence of an overall AChE excess (table 6, fig. 4). This confirms the significance of the effect noted above with the pectoral and PLD muscles *i.e.* that AChE biosynthesis is sensitive to a direct or indirect dystrophic gene effect in the embryo.

Conclusions.

There is a progression of molecular AChE forms in the development and growth of fast-twitch (type-IIB) fibres, leading to the accumulation of H_{2c} and its location in the endplates. As is logical, the series of enzyme profiles seen at the early developmental stages (Lyles, Silman and Barnard, 1979) strongly suggests that L_2 and M are precursors in the assembly *in vivo* of H_{2c}. In other fibre types, however, L and M forms can accumulate ; their function is as yet unknown. At maturity, ψChE occurs preferentially in the L_1 (monomeric) form, even in fast-twitch fibres ; its significance is also unknown.

The accumulation or assembly of these molecular forms appears to be determined by the respective muscle innervations. The formation of the endplate form of AChE (H_{2c}) at the time when innervation is proceeding has been shown by Kato *et al.* (1980) for mixed muscles of the embryonic leg. These effects may reflect a direct influence of the nerve or of induced electrical activity in the muscle. Evidence suggesting the latter comes from a study by Betz, Bourgeois and Changeux (1980) who, studying the effect of paralysing the ALD *in ovo* with Flaxedil, found that the 20 S form of AChE was much reduced, although endplates and their receptors were still present. The results reviewed here are consistent with this finding, but the nature of the neural influence still requires further study.

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Résumé. Les muscles du poulet présentent des avantages importants pour l'étude des intéractions nerfs-muscles, la cholinestérase servant de marqueur moléculaire. Plusieurs formes moléculaires de l'acétylcholinestérase (AChE) et de la pseudocholinestérase (ψChE) sont définies. Dans la plaque motrice du muscle rapide, l'AChE est sous la forme H_2C (20 S) ; elle possède un résidu terminal de collagène. Il en est de même pour la ψChE. L'évolution des différentes formes est décrite de façon quantitative, pendant le développement embryonnaire et la vie postnatale, dans plusieurs muscles comportant des types de fibres différentes chez le poulet normal et chez le poulet portant le gène de dystophie musculaire.

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