CHOLINESTERASES IN NORMAL AND DYSTROPHIC CHICKENS

by

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ABSTRACT

This work is a study of the enzymes acetylcholinesterase (AChE) and pseudocholinesterase (ψChE) in the skeletal muscle and blood of normal and genetically dystrophic chickens. The latter case, where abnormally high levels of AChE are known to occur, is of special interest. AChE exists in a series of molecular forms having sedimentation coefficients from 4S to 20S. ψChE occurs in a similar series of slightly smaller forms. The three smallest forms (4S, 7S, 11S) are globular proteins which are present in muscle and plasma; the heavier forms (15S and 20S) are asymmetric molecules which are present in muscle, but not in plasma. The progression of these forms during muscle development was established. The 20S form of AChE is of primary importance because it is considered to be specifically located at the neuromuscular junction. Its appearance in embryonic muscle was correlated with the development of the endplate. In normal fast twitch muscle *ex ovo*, 20S AChE remains the major form, but it eventually disappears in slow tonic muscle. Regulation of the synthesis of the various forms, thus, appears related to muscle fibre type. Results also shed light on their attachment at endplates.

The dystrophic lesion causes, specifically, an overproduction in muscle of the major light subunit of both enzymes, beginning by day 4 *ex ovo*; this later leads to excesses of the heavier forms, as well as increased AChE in plasma. No excess ψChE appears in the blood. These data were applied in assessing the chemotherapy of dystrophic chickens: the release of excess AChE into the blood and, in favourable cases, its overproduction in muscle were partially suppressed by certain drugs.

These studies support the model of AChE biosynthesis whereby larger oligomers are formed by a sequential association of monomeric (4S) subunits. ψChE may be synthesized similarly, since these studies show several significant similarities between ψChE and AChE.

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PREVIOUS PUBLICATIONS

Some of the information contained in this thesis has been published in previous papers:

1. Silman, I., J.M. Lyles and E.A. Barnard (1978) "Intrinsic forms of acetylcholinesterase in skeletal muscle." FEBS. Letts., 94, 166-170.

2. Silman, I., L. DiGiamberardino, J.M. Lyles, J.Y. Couraud and E.A. Barnard (1979) "Parallel regulation of acetylcholinesterase and pseudocholinesterase in normal, denervated and dystrophic chicken skeletal muscle." Nature (Lond.), 280, 160-162.

3. Lyles, J.M., I. Silman and E.A. Barnard (1979) "Developmental changes in levels and forms of cholinesterases in muscles of normal and dystrophic chickens." J. Neurochem, 33, 727-738.

4. Lyles, J.M. and E.A. Barnard (1980) "Disappearance of the 'endplate' form of acetylcholinesterase from a slow tonic muscle." FEBS. Letts., 109, 9-12.

5. Lyles, J.M., E.A. Barnard and I. Silman (1980) "Changes in the levels and forms of cholinesterases in the blood plasma in normal and dystrophic chickens." J. Neurochem, 34, 979-988.

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ABBREVIATIONS

Absorbance	=	A
Acetyl-β-methylcholine	=	MeCh
Acetylcholine	=	ACh
Acetylcholinesterase	=	AChE
Acetylthiocholine (iodide)	=	AThCh(I)
Alcohol dehydrogenase	=	ADH
Anterior latissimus dorsi	=	ALD
Butyrylcholine	=	BuCh
Butyrylthiocholine (iodide)	=	BuThCh(I)
Cholineacetyltransferase	=	AcCh
Creatine phosphokinase	=	CPK
Day	=	d
5,5'-dithiobis-2-nitrobenzoic acid	=	DTNB
Flexor carpi ulnaris	=	FCU
Incubation factor	=	IF
Lactate dehydrogenase	=	LDH
Minute	=	min.
Molecular weight	=	MW
N-ethylmaleimide	=	NEM
Optical density	=	OD
Posterior latissimus dorsi	=	PLD
Proprionylcholine	=	PrCh
Pseudocholinesterase	=	ψChE
Pyruvate kinase	=	PK
Sedimentation coefficient	=	S in Svedberg units ($s_{20,w}$)
Succinic dehydrogenase	=	SDH
Tetraisopropylpyrophosphoramide	=	isoOMPA
Unit (of enzyme activity)	=	U

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THE AIM OF THE THESIS

The aim of this thesis is to characterize AChE and ψChE in the chicken, especially in the dystrophic chicken where abnormal control causes excess levels of both enzymes. Previous studies by gel electrophoresis did not detect all the enzyme forms which are present in muscle. By employing sucrose gradient velocity sedimentation in this study, particular attention will be given to the 20S, presumably endplate-specific, form. ψChE has not been characterized well in muscle, in general. Therefore, the synthesis of ψChE forms and a comparison with AChE will be studied in order to elucidate the relationship between these two enzymes.

Changes in the levels of the forms of AChE and ψChE will be examined in fast twitch and slow tonic muscles and plasma during embryonic development and further maturation in order to determine:

1) Does the order of appearance of the different forms _in ovo_ give any indication of the _de novo_ synthesis of the enzymes?
2) Is there any correlation of particular forms with different types of muscles?
3) What is the relationship of the enzyme forms present in plasma and muscle?

The dystrophic chicken provides an unusual model for the study of AChE and ψChE. It is hoped that this study of the abnormal control of the enzymes will also elucidate their normal synthesis and relationship. Dystrophic chickens will be examined, further, to define the exact nature of the effect of the abnormal gene on AChE and

ψChE and to determine when the gene first begins functioning. The knowledge gained from this study will be applied in the analysis of the effects of chemotherapy on these enzymes in muscle and plasma of dystrophic chickens.

CHAPTER 1

INTRODUCTION

1.1 Acetylcholinesterase

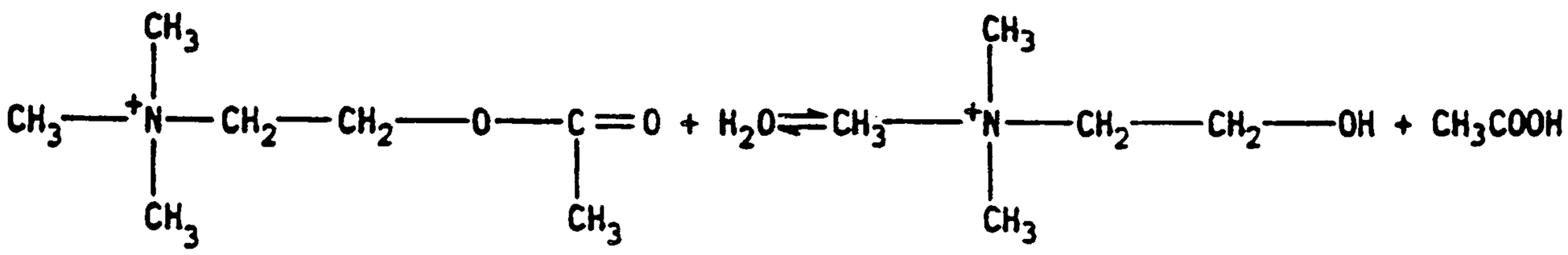
1.1.1 Characterization

Acetylcholinesterase (AChE, EC 3.1.1.7) has been considered to be important in muscle contraction ever since Marnay & Nachmansohn (1938) proposed that acetylcholine (ACh) is a neurotransmitter and illustrated that AChE is concentrated at the nerve endings of skeletal muscles. An examination of the location, structure and synthesis of AChE is necessary in order to understand how this enzyme functions in muscle. First, a general description of its enzymic characteristics will be given.

Cholinesterases catalyze the hydrolysis of ACh yielding acetic acid and choline(Fig.1A). This class of enzymes has been classified on the basis of substrate specificity in vitro (Mendel et al, 1943; Nachmansohn & Rothenberg, 1945; Ord & Thompson, 1952). "True" cholinesterase (AChE) usually hydrolyzes the choline esters ACh and acetyl-β-methylcholine (MeCh) faster than butyrylcholine (BuCh) or other acyl esters. In the chicken, AChE is different to that in mammalian species in also showing a high affinity for propionylcholine (PrCh; Blaber & Cuthbert, 1962). A second cholinesterase present in muscle is pseudocholinesterase (ψChE, EC 3.1.1.8) which usually hydrolyzes PrCh, BuCh or benzoylcholine faster than ACh. "Non specific" esterases hydrolyze, in addition to the substrates mentioned, aliphatic and non-choline esters and glycerides.

A distinctive feature of AChE, not exhibited by ψChE, is its inhibition at high substrate concentrations (Augustinsson et al, 1952): it can be distinguished by its activity at low ACh concentrations and its inactivity at high substrate concentrations. AChE can further be distinguished from other cholinesterases by using special AChE inhibitors: 1) the specific inhibitor 1,5-bis(4-allyl-dimethylammonium-

A. Hydrolysis of ACh



Acetylcholine **Choline** **Acetic acid**

B. Hydrolysis of AThCh -- the Ellman reaction

1. Acetylthiocholine $\xrightarrow{\text{enzyme}}$ Thiocholine + Acetate

2. Thiocholine+5:5-dithiobis-2-nitrobenzoate $\longrightarrow$

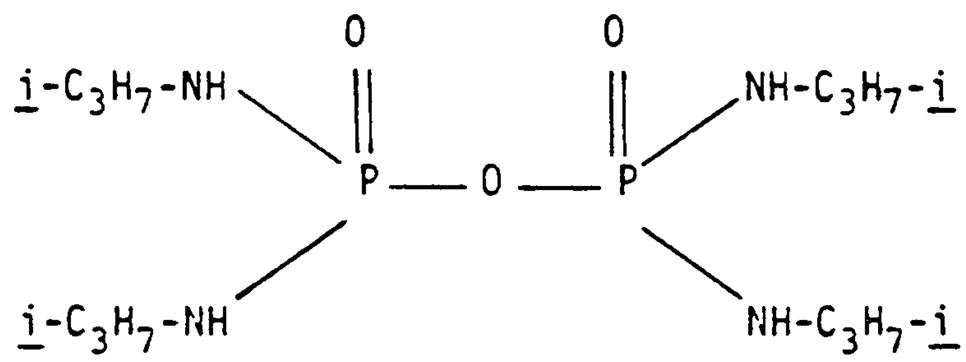
5-thio-2-nitrobenzoate (yellow coloured) &

mixed salt of thiocholine and 5-thio-2-nitrobenzoic acid.

Figure 1. Reactions catalyzed by AChE.

A. isoOMPA (ψChE inhibitor)

iso-OMPA; DPDA — Tetramono*iso*propylpyrophosphortetramide; *N,N'*-di*iso*propylpyrophosphorodiamidic **anhydride**



B. BW284C51 (AChE inhibitor)

BW284C51 — 1:5-bis (4-allyldimethylammoniumphenyl)-pentan-3-one dibromide

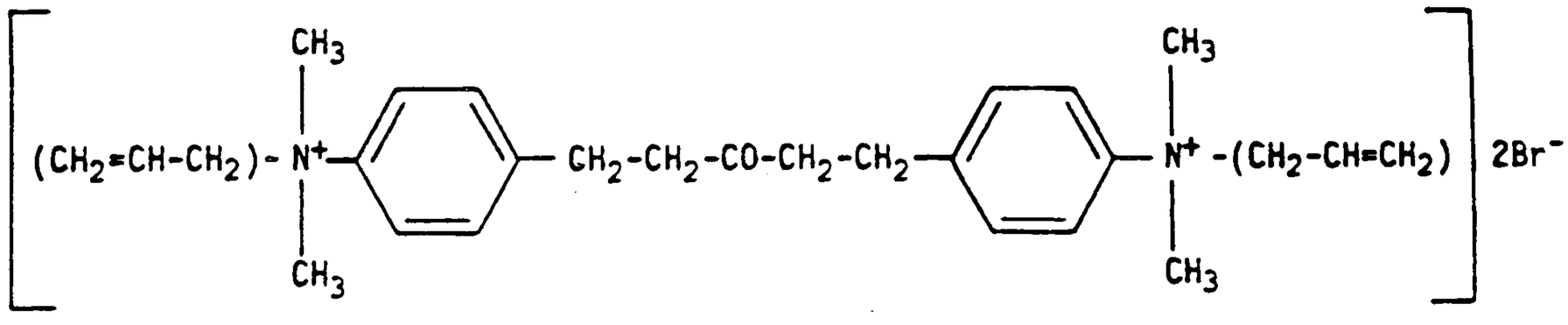


Figure 2. Structures of specific inhibitors of AChE and ψChE.

phenyl)-pentan-3-one-dibromide(BW284C51; Fig.2) and 2) high (10^{-5} - 10^{-6}M) concentrations of diisopropylphosphofluoridate (DFP). BW284C51 is a reversible, competitive inhibitor of AChE; DFP is an irreversible inhibitor which is specific for AChE when used at high concentrations, but it also inhibits ψChE when used at lower concentrations (for general reviews of inhibition see:Silver, 1974; Rosenberry, 1975). See Table 1 for a summary of the differences between AChE and ψChE.

1.1.2 Localization in tissues

Regardless of the various substrates utilized in vitro, AChE in vivo hydrolyzes ACh which is released from nerve terminals during neurotransmission. Thus, AChE is abundant in brain and skeletal muscle where it is found by histochemical staining in high concentrations at cholinergic nerve endings (Koelle & Friedenwald, 1949; Denz, 1953; Bonichon, 1957; Gerebtzoff, 1959; Lehrer & Ornstein, 1959). More specifically, AChE has been located on the prejunctional and postjunctional membranes of nerve terminals (Davis & Koelle, 1967) and of muscle endplates; in the latter case it is mostly on the postjunctional membrane (Barnett, 1962). AChE is attached to the basal lamina of the postsynaptic cleft of the endplate (McMahan et al, 1978). The synaptic cleft has upper, primary folds and deeper, secondary folds. In contrast to the ACh receptors which are localized on the primary folds, AChE covers both primary and secondary folds. This arrangement may enable a high concentration of AChE in relation to receptors at endplates where there is a high level of ACh release. Due to its proximity to the receptors, AChE is ideally situated to function in the removal of ACh from receptors during the process of neurotransmission.

The presence of AChE in other tissues varies, depending on the species, but includes serum (Stedman & Stedman, 1935) and various membranes, such as in erythocytes, platelets, gut, placenta and blood

TABLE 1

A COMPARISON OF THE CHAKACTERISTICS

OF AChE AND ψChE

	AChE	ψChE
Substrate preference		
In general	ACh, MeCh	PrCh>BuCh,ACh
Especially in the chicken	also PrCh	also MeCh
Substrate inhibition	Yes	No
Specific inhibitors	BW284C51 DFP - high concentrations	isoOMPA DFP - low concentrations Ethopropazine
Subunit sizes (S)		
Eel[a]	5,7,11; 9,14,18	-
Rat[b,c]	4,6,10; 9,13,16	4,7,11,17
Human[b]	4,6,11; 10,13,17	-

[a]Bon et al, 1976

[b]Bon et al, 1979

[c]Vigny, et al, 1978

Based on substrate preferences the second cholinesterase in the chicken is more appropriately called pseudocholinesterase (ψChE) than butyrylcholinesterase (the latter name is commonly used for the enzyme in mammalian species).

vessel walls (Zajicek et al, 1954; Gerebtzoff, 1959; Silver, 1974). Although its exact role in these tissues is unclear, this investigation is concerned primarily with the enzyme involved in cholinergic muscle.

1.1.3 Molecular structure

Early chromatographic analysis indicated that AChE is an isozymal complex (Bernsohn et al, 1961; Leuzinger et al, 1969). AChE, a glycoprotein, has been confirmed as having an oligomeric structure in the many species and types of tissues examined. The absolute subunit sizes vary with the species, but similar series of molecular weights (MW) are found universally (see Table 1; also Leuzinger et al, 1969; Massoulié & Rieger, 1969; Chan et al, 1972; Chen et al, 1974; Silver, 1974). The structure of AChE from the electric organ of Electrophorus electricus, a rich source of the enzyme, has been characterized very well. Although the major, native form of AChE in Electrophorus is the largest one ($s_{20,w}$ = 18S, Svedberg units; 1,150,000 MW), some 14S AChE is also present (Grafius & Millar, 1965). These large forms can be degraded to smaller subunits of 280,000 MW, 160,000 MW and 80,000 MW. The 280,000 MW form (11S) is a tetramer which has four identical catalytic subunits, one on each monomer (Kremzner & Wilson, 1964; Chen et al, 1974). The tetramer has inter-subunit disulfide bonds which join two 6.2S (ca 150,000 MW) dimers; disulfide bonds also join two 3.8S monomers (ca 75,000 MW) in the dimer (Chen et al, 1974; Bon & Massoulié, 1976).

Massoulié et al (1971) proposed that Electrophorus AChE exists in two basic forms: globular (4S, 6S, 11S) and asymmetric (8S, 14S, 18S). The globular forms are monomer, dimer and tetramer. The others are asymmetric because they possess a tail which is collagenous (as also shown by: Rosenberry & Richardson, 1977;

Anglister & Silman, 1978). Addition of one, two or three 11S tetramers to the tail yields, respectively, the 8S, 14S and 18S forms. Forms larger than 18S which were detected at the bottom of sucrose gradients following velocity sedimentation are probably aggregates of several asymmetric forms that are cross-linked via their collagen tails (Dudai et al, 1973).

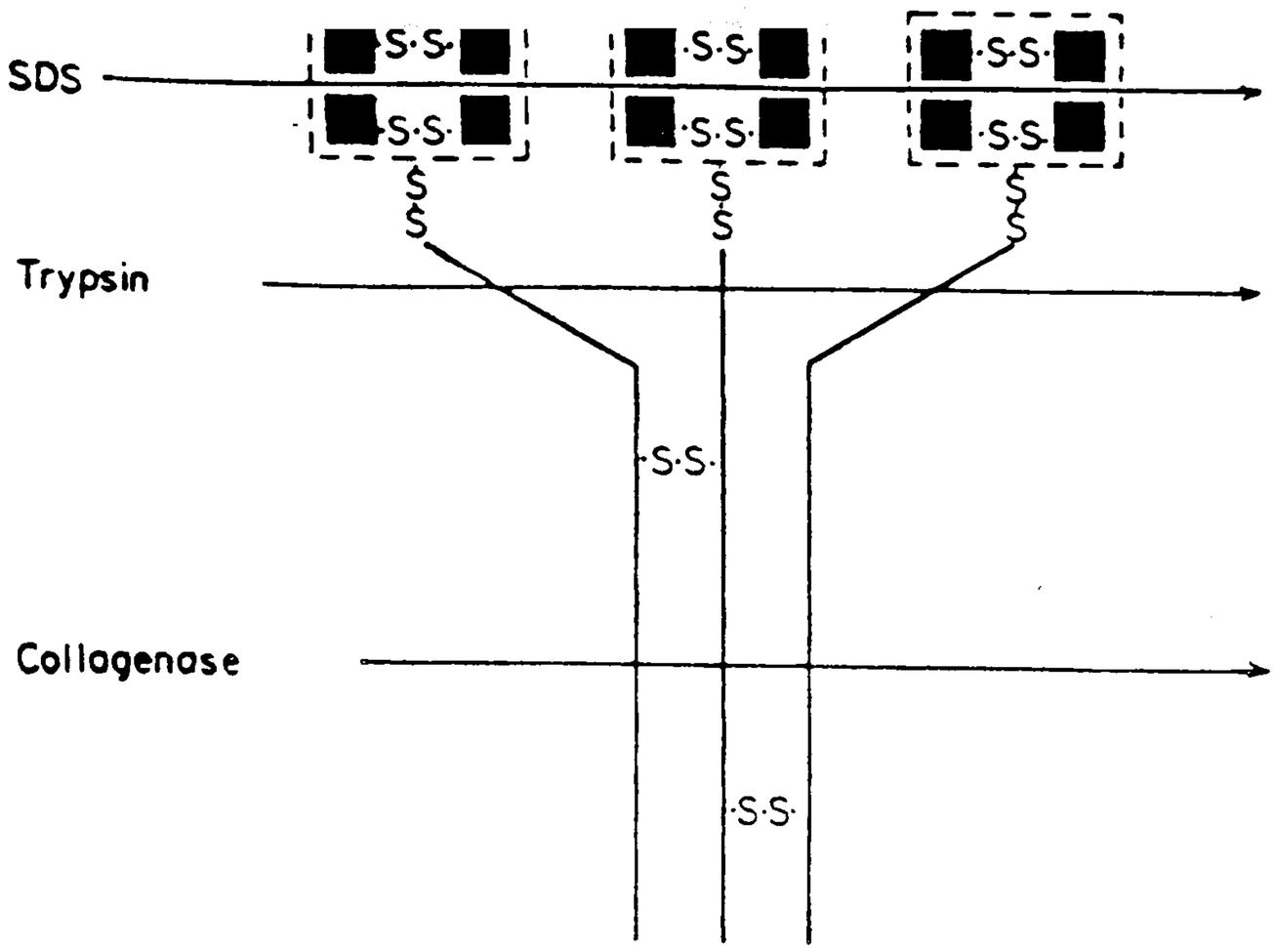
The asymmetric forms are less soluble than the globular forms and are not easily extracted from tissue in the absence of high salt concentrations and Triton X-100, (Dudai & Silman, 1974a). Therefore, they were not detected in early work on chicken muscle by Wilson et al (1969) because aqueous extraction was used. In addition, the method of analysis (polyacrylamide gel electrophoresis) used in those studies does not detect forms much larger than 10-11S because they cannot penetrate the gel (Bon & Massoulié, 1976). A direct comparison of rat AChE forms (4S, 6.5S, 10S) detected by gel electrophoresis and by sucrose gradient sedimentation shows that the slowest electrophoretic band corresponds to the 10S form, the fastest band corresponds to the 4S form, but the 6.5S form is represented by several intermediate electrophoretic bands (Gisiger et al, 1978). Therefore, data on AChE forms in chicken muscle which employ gel electrophoresis do not accurately represent all of the forms in muscle or their true subunit sizes. Sucrose gradient velocity sedimentation, however, can detect forms much larger than 10S and can separate each form more clearly on the basis of size.

A 20S form, as well as 4S, 6S and 11S forms, was detected in chick ciliary ganglia by employing high salt/Triton X-100 extraction and sucrose gradient velocity sedimentation (Vigny et al, 1976a). Thus, AChE exists in the chicken in a series of subunit sizes which fits the proposed structural model of AChE in Electrophorus.

A recent direct comparison of the subunit sizes of Electrophorus, rat, bovine and chicken AChE by Bon et al, (1979) shows that the proposed structural model appears to hold for AChE, generally, in many species (see Table 1).

Electron microscope pictures of the larger forms of AChE (Dudai et al, 1973, Rieger et al, 1973a) further support this model by revealing a structure which has a rod-like tail and several, usually three, globular heads. The tail is believed to be a triple helix of collagen with one tetramer attached to the end of each collagen strand (Anglister & Silman, 1978; Rosenberry & Richardson, 1977; see Fig.3). McMahan et al, (1978) have proposed that the tail anchors AChE to the basal lamina (which is also collagenous) of the postsynaptic membrane. A further indication that a collagenous component attaches AChE to the synaptic membrane is that collagenase treatment specifically increases the amount of AChE extracted from muscle (Sketelj & Brzin, 1977; Sung, 1978) while not disrupting the membrane structure (Sketelj & Brzin, 1979). Collagenase treatment converts purified asymmetric forms to lighter, less asymmetric forms by cleaving the tail in the middle, thus leaving the three tetramers linked by a short piece of tail (Bon & Massoulié, 1978; Anglister & Silman, 1978). Trypsin treatment also degrades the asymmetric forms. Apparently this enzyme attacks the tail close to its connection with the tetramers, sequentially releasing tetramers from the asymmetric forms: 18S→14S(+11S)→8S(+11S)→11S (Vigny et al, 1979a).

The other area of the AChE molecule about which structural information is known is the active site (for general reviews see Silver, 1974; Rosenberry, 1975). This is composed of two sites: anionic and esteratic. The anionic site attracts the choline moiety of the substrate, ACh. The esteratic site contains an acidic group



A.

B.

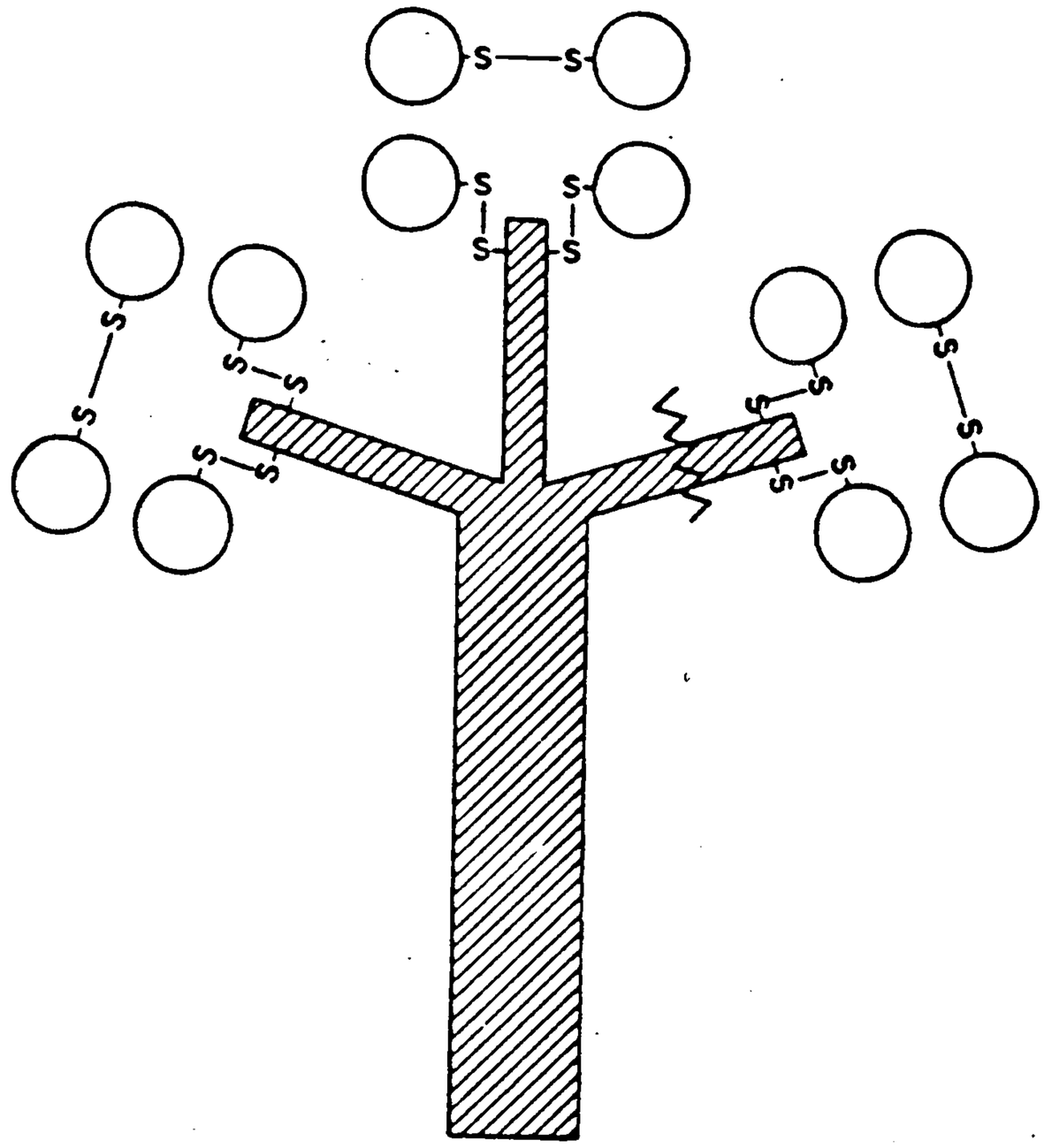


FIGURE 6: Schematic model of 18S eel acetylcholinesterase. The circles refer to catalytic subunits, and the cross-hatched region, to an as yet undefined combination of polypeptides E which comprise the tail structure. Partial cleavage of catalytic subunits is ignored. Catalytic subunits may be considered in dimeric pairs. In half of these pairs, subunits are disulfide linked directly to each other, while in the other half, a disulfide linkage is mediated via the tail subunits. Both a 14S and an 11S species are generated from an 18S molecule by cleavage at the indicated tail structure site.

Figure 3. Structural models of AChE

(A) From Anglister & Silman, 1978
(B) From Rosenberry & Richardson, 1977
In both models, a tail is envisaged as joining three tetrameric heads by disulphide bonds. Catalytic subunits (monomers) are represented as either squares (A) or circles (B). The proposed sites of action of SDS, trypsin or collagenase are indicated in (A) by arrows.

which attracts the acetyl moiety of the substrate, and a basic group which joins the acetyl group to the enzyme. Another basic group may be involved in deacetylation which occurs when water reacts with the complex, releasing acetic acid and choline. In addition, molecules may act at an allosteric site which could alter the conformation of the molecule, thereby altering the reactivity of the active site (Changeux, 1966). Specific inhibitors act by combining with either the anionic site, the esteratic site or both, thus blocking the entry or the hydrolysis of substrate.

1.1.4 Synthesis

Although the membrane localization of AChE at the muscle endplate is well established, less is known of the internal sites or the synthesis of AChE. In neurons, AChE is believed to be synthesized in the cell body and transported down the axon to the nerve terminal where it appears in vesicles and on the axoplasmic membrane (Fukuda & Koelle, 1959; Silver, 1974; Di Giamberardino & Couraud, 1978; Davis & Koelle, 1978; Wooten & Cheng, 1980).

Although it has been debated whether or not the nerve is the source of AChE in muscle, this appears unlikely for several reasons. First, embryonic myoblasts and myotubes produce AChE, which is distributed diffusely in the cytoplasm, at a stage before innervation has occurred (Mumenthaler & Engel, 1961; Tennyson et al, 1973). Second, such myoblasts continue to produce AChE when grown in tissue culture in the absence of nerves (Goodwin & Sizer, 1965; Shainberg et al, 1971; Fluck & Strohman, 1973; Sawyer et al, 1976). Third, although muscles in dystrophic chickens have abnormally high AChE levels, the levels and rate of transport of AChE in their motor neurons is normal (Di Giamberardino et al, 1979).

Internal sites of staining for AChE in muscle cells are the endoplasmic reticulum, sarcotubules and nuclear envelope (Mumenthaler & Engel, 1961; Miledi, 1964; Ulbrecht & Kruckenberg, 1965; Tennyson et al, 1973; Silver, 1974). Following AChE inhibition in tissue-cultured chicken muscle cells, Wilson & Walker (1974) found that newly synthesized AChE first appears in the cytoplasm and it is only the "light" form (as detected by electrophoresis). Stain later appears at the cell surface when "heavier" (probably 11S) forms are detected. This implies that AChE forms are synthesized sequentially: smaller forms, synthesized on the endoplasmic reticulum, may be incorporated into larger ones which are assembled at or migrate to the cell surface, possibly via the sarcotubules. Such a process is also suggested by the order of appearance of AChE staining sites during embryonic development of muscle cells (Mumenthaler & Engel, 1961). General cytoplasmic staining occurs in the myoblast around day(d)6 in ovo. As differentiation into myotubules proceeds stain appears in regular transverse lines and becomes concentrated in the area of the future neuromuscular junction. When innervation occurs, AChE finally appears localized at endplates, around d14 in ovo, and cytoplasmic stain decreases. Thus, AChE is synthesized inside the muscle fibre and transported, possibly via the internal membranes, to its external location on the postsynaptic membrane.

1.2 Pseudocholinesterase

1.2.1 Characterization

ψChE is a second cholinesterase which occurs in muscles and plasma and is of interest in this study because of its many similarities to AChE. (For a general review, see Silver, 1974). Like AChE, it hydrolyzes ACh. In addition, it hydrolyzes BuCh (at a similar rate to

ACh) and PrCh (at an even greater rate). ψChE in the chicken is different than ψChE in other species in that it also hydrolyzes MeCh, a substrate usually considered specific for AChE (Blaber & Cuthbert, 1962). Unlike AChE, ψChE can also utilize aliphatic and aromatic esters (e.g. tributyrin and phenyl b utyrate). ψChE is not inhibited by high substrate concentrations. On the contrary, ψChE utilizes ACh more effectively at high concentrations as indicated by its higher K_m, while AChE is inhibited under such conditions.

ψChE can be differentiated further from AChE by using specific inhibitors, such as tetra*iso*propylpyrophosphoramide (isoOMPA) and ethopropazine-HCl (Fig.2), and, also, by selectively employing DFP at lower concentrations than those needed to inhibit AChE (e.g. 10^{-7}M). IsoOMPA and DFP are irreversible inhibitors, whereas ethopropazine is a reversible inhibitor.

1.2.2 Localization in tissues

ψChE, like AChE, is a glycoprotein. The plasma-derived enzyme is water soluble; the tissue-derived enzyme, although less so, is still more soluble than AChE. ψChE is found in heart and brain (Ord & Thompson, 1952), pancreas and plasma (Mendel & Rudney, 1943), and liver and gut (Gerebtzoff, 1959). The source of ψChE in plasma is probably the liver, which is known to synthesize ψChE, as well as other plasma enzymes (Svensmark, 1963). The exact roles of ψChE in the variety of tissues where it is found have not been defined. In the gut it may be involved in food assimilation (Gerebtzoff, 1959) and its occurence in plasma and the pancreas may be related to this role. Its presence in fat cells of the obese mouse (Kutty *et al*, 1979) suggests that it may play a role in fat metabolism. In the brain, ψChE is concentrated in the white matter of the cerebrum which led Ord & Thompson (1952) to suggest that its role in the nervous system

is related to processes in the myelin sheath or neuroglial cells rather than to neurotransmission itself.

The role of ψChE in muscles, however, appears to be related to neurotransmission since, like AChE, it is localized on the synaptic membranes of endplates in several species, although also present in Schwann cells (Holmstedt, 1957; Davis & Koelle, 1967; Silver, 1974). ψChE is present at skeletal muscle endplates in much smaller amounts than AChE. Likewise, the total amount of ψChE activity in aqueous extracts of embryonic and adult chicken muscle is much less than the amount of AChE activity (Maynard, 1966; Wilson et al, 1973a). The role of ψChE at the neuromuscular junction may, therefore, be a supportive one. It may assist AChE in hydrolyzing ACh if abnormally high substrate concentrations occur since such conditions may inhibit AChE but allow for optimal ψChE activity (Silver, 1974).

A different role for ψChE in the cat superior cervical ganglia was proposed by Davis & Koelle (1978). Since they detected ψChE only postsynaptically (not presynaptically), they proposed that it is a precursor in the synthesis of synaptic AChE. Later Koelle et al (1979) showed that AChE levels in vivo decrease following the selective inhibition of ψChE. However, since the effect of such inhibitors on all aspects of cell metabolism are not clear, their argument is not definitive. Furthermore, they were studying neurons, which may not regulate ψChE in the same way as muscles.

1.2.3 Molecular structure

ψChE, like AChE, occurs in multiple forms. Early studies using starch gel electrophoresis or paper chromatography were done on ψChE from tissues where enzyme is plentiful and soluble, such as

plasma or pancreas (Mendel & Rudney, 1943; Bernsohn et al, 1961; Harris et al, 1962; La Motta et al, 1965). La Motta et al showed that plasma ψChE occurs as four smaller forms which can all be converted into the major, largest form (ChE5); they proposed that the conversion occurs by a stepwise polymerization. Two larger forms were found (La Motta et al, 1968) which could be converted into the more mobile forms and these were considered to be conformational forms of ChE5. They determined the molecular weights of several forms by polyacrylamide gel electrophoresis: 82,000, 110,000, and 260,000 (La Motta et al, 1970). Of four apparent isozymes purified from human serum, the largest was found to be 366,000 MW (Das & Liddell, 1970). Thus, the series of molecular weights of the ψChE forms is very similar to the sizes of AChE forms. These plasma-derived forms probably represent only the soluble, globular ones, since the tailed forms are larger, membrane-bound and hard to solubilize.

Recently, multiple forms of ψChE have been detected in rat muscle and sympathetic ganglia (Vigny et al, 1978). The sizes of ψChE forms are similar to the series of AChE forms which occur in the rat, only each ψChE form is ca 0.5S larger than the corresponding AChE form (see Table 1). Thus, a large ψChE form exists which corresponds to the largest, tailed form of AChE. It may likewise possess a tail with which it could be anchored at the synapse. Several bands of ψChE activity (smaller than 11S) are also detected on gel electrophoresis of plasma and muscle from chickens (Wilson et al, 1973b). But, as previously described (1.1.3), these studies did not allow the detection either of forms larger than 11S or of the less soluble forms.

The basic structure of ψChE at the active site is thought to be similar to that of AChE (i.e. there are esteratic and anionic sites;

Silver, 1974). The differences between the two enzymes regarding substrate and inhibitor specificities may be due to small differences in the structure of the active site and, also, possibly of neighbouring areas.

1.2.4 Theories accounting for similarities of ψChE and AChE

The similarities between ψChE and AChE might be considered as confirming Koelle et al's proposal that ψChE is a biosynthetic precursor of AChE. Vigny et al (1978) attempted to disprove this theory concerning the enzymes in the rat by showing differences in thermal inactivation, no immunological cross reactivity of ψChE with antisera specific for AChE and, also, no corresponding decrease in one enzyme when there was an increase in the level of the other. They concluded that these two enzymes are distinct products of different genes. Their immunological argument does not settle the question completely since the exact immunogen on AChE is unknown and could also exist on ψChE but be masked due to conformational or other structural differences. Also, very little is understood of the metabolism or regulation of these enzymes so that their final argument is not definitive.

To account for the similarities both between these two enzymes and, also, between cholinesterases of various species, Augustinsson (1948) proposed that cholinesterases originated from the same gene but have diverged during evolution: from their early homogeneous form in lower vertebrates and fish to their more complex forms, with different substrate and inhibitor specificities, in higher vertebrates. This is a reasonable hypothesis which could account for both the existence of the similar series of forms of AChE and ψChE and their different absolute sizes and enzymic characters.

1.3 Striated Muscle Fibre Differentiation

1.3.1 Adult fibre differentiation

1.3.1.1 Physical differences

Two types of striated muscle fibre in the chicken, slow tonic and fast twitch, have extremely different characteristics. The anterior latissimus dorsi (ALD) muscle is composed almost purely of slow tonic fibres, while the pectoralis (pectoral) and posterior latissimus dorsi (PLD) muscles consist almost exclusively of fast twitch fibres. The two fibre types are easily distinguished by their patterns of innervation. Slow tonic fibres are multiply innervated by small axons, whereas fast twitch fibres are singly innervated by large axons (Ginsborg & Mackay, 1960; Hess, 1961). Slow tonic fibres are slightly larger than fast twitch fibres (Fedde, 1969) and more red in colour due to the presence of myoglobin; they also have a greater vascular bed and a faster rate of blood flow (Hudlicka, 1969). Other physical differences relate to the internal and cellular organization of the fibres (Hess, 1961 &1967). The Z disc runs straight across fast twitch fibrils, whereas it is more zigzagged in slow tonic muscles. Fast twitch fibres have a well developed, while slow tonic fibres have a reduced, sarcoplasmic reticulum. Fast twitch fibres have a T-system, whereas slow tonic fibres do not.

The endplates are also distinctive in the two types of fibres (Ginsborg & Mackay, 1960; Hess, 1967). The endplates on fast twitch fibres occur singly (one per fibre) and all called "en plaque". They are very prominent, defined at their borders by the subneural apparatus, and are much larger than slow tonic fibre endplates. The junctional folds of "en plaque" endplates in the chicken, although shorter and less numerous than those in fast twitch fibres of mammals

and other species, are, nonetheless, greater than those of slow tonic fibre endplates. The latter type of endplate is called "en grappe", meaning clustered, and occurs multiply on slow tonic fibres (ca 80 per ALD fibre). They are less complex than "en plaque" endplates. A collection of small, dot-like shapes having no well developed interconnecting network of channels, they lie flat on the fibre surface and lack junctional folds. "En grappe" endplates are smaller and are innervated by smaller axon terminals which release less AChE than in "en plaque" endplates. Acetylcholinetransferase (AcCh, Acetyl-CoA: choline O-acetyltransferase, EC 2.3.1.6; an enzyme necessary in the synthesis of ACh) and AChE are also present in smaller amounts at "en grappe" endings (Buckley & Heaton, 1971: O'Brien & Vrbová, 1978).

1.3.1.2 Functional differences

Slow tonic muscles, as the name implies, exhibit slower contraction and relaxation times than fast twitch muscles (Page, 1969). These functional patterns are correlated to differences in membrane characteristics of the fibres (Vrbová et al, 1978). Fast twitch fibres are sensitive to ACh only at the single area of the endplate and their membranes are well developed to conduct action potentials. Their nerves show a higher frequency of electrical discharge that usually elicits a propagated action potential along the muscle fibre which causes a fast, twitch type of contraction. Slow tonic fibres are more sensitive to ACh than fast twitch fibres at multiple sites (i.e. the endplates) and, also, extrajunctionally. The membrane of slow tonic fibres is not well developed to conduct action potentials. Their nerves fire at a slower frequency which does not often elicit an action potential and such action potentials are rarely propagated. Stimulation of slow tonic muscles causes local action potentials at multiple sites along the fibre which results in a slow, graded

contraction. The recovery time is also slower than in fast twitch fibres. Thus, the physical and functional differences of these fibre types enable the sudden and co-ordinated contraction of fast twitch fibres in voluntary muscles, as contrasted with the slower and maintained contraction of slow tonic fibres which is required in postural muscles.

1.3.1.3 Biochemical differences

The underlying mechanism which enables slow or fast muscle contraction is biochemical. First, different myosin light chains have different ATPase activities which determine the speed of contraction; slow tonic and fast twitch fibres have different myosin light chains (as first shown in the rabbit by Bárány et al, 1965). Second, the two muscle fibre types derive energy via different metabolic pathways (Bass et al, 1969). Slow tonic muscles, as indicated by a greater supply of O_2 (e.g. myoglobin content, vascular nature, and rate of blood flow), rely on oxidative metabolism. They, therefore, contain high levels of mitochondrial enzymes (e.g. succinic dehydrogenase, SDH, EC 1.3.99.1). Fast twitch muscles require an alternative means of energy production (i.e. anaerobic metabolism) and possess enzymes necessary for glycolysis, glycogenolysis and lactic acid fermentation (e.g. lactate dehydrogenase, LDH; L-lactate: NAD oxidoreductase, EC 1.1.1.27; or phosphorylase, EC 2.4.1.1.) Thus, slow tonic muscles are equipped metabolically to produce energy slowly but for a longer period, as needed to sustain a continuous contraction, whereas fast twitch fibres can produce larger amounts of energy more rapidly, as required for short periods of sudden contraction.

1.3.2 Embryonic fibre differentiation

1.3.2.1 Myoblast

The myoblast is the earliest stage in muscle cell development. (For general reviews see: Mumenthaler & Engel, 1961; Vrbová et al, 1978.) It is a largely undifferentiated, mononucleated cell which undergoes a series of mitoses. Following the final mitosis, the myoblast synthesizes several proteins specific to muscle cells: contractile proteins (myosin, actin and tropomyosin), ACh receptors, and AChE. AChE is distributed diffusely in the cytoplasm at this stage.

1.3.2.2 Myotube

Multinucleate myotubes are formed by the fusion of myoblasts. They continue to synthesize special muscle proteins; contractile proteins form a banded pattern in the cytoplasm; sarcoplasmic reticulum is elaborated; ACh receptors appear over the entire surface membrane; and AChE increases throughout the cytoplasm, although it also appears along transverse lines. Embryonic myoblasts differentiate into myotubes when grown in tissue culture and they show a similar increase in AChE synthesis (Goodwin & Sizer, 1965). Several forms of AChE (probably 4S, 7S, 11S), as determined by polyacrylamide gel electrophoresis, are found at this stage (Wilson & Walker, 1974). Thus, the necessary contractile apparatus is present at the myotube stage to enable the spontaneous contractions which are observed in vitro and at d3-4 in ovo. Although exploratory nerves are present in myotomes at this stage, there are not yet any real nerve contacts with muscle cells (Filogamo et al, 1978).

1.3.2.3 Myofibre

Myotubes rapidly develop into myofibres when nerve terminals establish contact with them, causing synapse formation and triggering a

series of other biochemical changes. The exact stage of this differentiation *in ovo* varies with the specific muscle, ranging from d7-17: ALD and PLD muscles become innervated between d9-13, while it may occur several days later in the pectoral muscle (Hirano, 1967; Bennet & Pettigrew, 1974; Atsumi, 1977; Ashmore *et al*, 1978; Vrbová *et al*, 1978). The innervation pattern of a particular myofibre is determined by the type of nerve which first contacts it at the myotube stage: slow motoneurons cause multiple innervation, while fast motoneurons cause single innervation.

Initial nerve contact is followed by a rapid proliferation of ACh receptors (Giacobini *et al*, 1973) and of AChE. The latter is localized at the synpase two to three days after nerve contact and, along with ACh receptors, is called the subneural apparatus. Histochemical staining for AChE increases progressively at the neuromuscular junction after this point in development, while decreasing in the cytoplasm . The first actual particles which stain for AChE and correspond to its endplate localization were found to occur as small, discrete rod-shapes on d14 *in ovo* in the paravertebral muscle (Mumenthaler & Engel, 1961). The same three forms of AChE as detected by electrophoresis during the myotube stage occur in the myofibre. "En grappe" endplates, showing much less stain for AChE, are different than "en plaque" endplates, which have more AChE, from the earliest stage when they are detected (e.g. d14-15 in ALD and PLD muscles; Gordon *et al*, 1977).

Once endplates are established, biochemical and functional differentiation occurs which further distinguishes the fibre types. Membrane sensitivity to ACh becomes more restricted to the endplate regions-- to single areas of fast twitch fibres and to multiple areas on slow tonic fibres. Metabolic enzymes increase and differentiate:

more SDH is apparent in slow tonic ALD fibres on d16 *in ovo*, while more phosphorylase is present in fast twitch PLD fibres (Gordon *et al*, 1977). Although these enzyme differences are not established permanently until final differentiation *ex ovo*, they indicate that biochemical changes precede function differences; a faster speed of contraction and lower membrane resistance are not detected in the fast twitch PLD until d17 *in ovo* (Gordon *et al*, 1977). The different types of myosin, however, do not differentiate until after hatching.

Endplates develop further during later embryonic growth - the "en plaque" type becomes larger and more complex, while "en grappe" endplates are smaller and show less histochemical stain for AChE. AChE intensifies at the endplates and disappears completely from the cytoplasm. Although 4S, 7S and 11S AChE were detected at this stage, the study of AChE forms in embryonic chick muscle is incomplete because electrophoretic studies did not detect the highly important, presumbably endplate-specific, 20S form (as explained in 1.1.3). In embryonic rat muscle, the largest (16S) form of AChE appears in myofibres later in embryonic development and, also, in myofibres which develop endplates when grown in the presence of nerves in tissue culture (Koenig & Vigny, 1978a). In the chicken, the lighter forms of AChE are lost during further development *ex ovo* in the fast twitch muscles while they are retained in the slow tonic ALD muscle. The timing of these changes and the initial appearance of the 20S form, however, have not been established.

Further differentiation of the two fibre types occurs during the first several weeks *ex ovo*. Fast twitch fibres stop synthesizing the enzymes for aerobic metabolism (Bass *et al*, 1970).

The different types of myosin also differentiate during this period (Gauthier et al, 1978) and the different electrical and functional characteristics are established.

1.4 Muscular Dystrophy in the Chicken

1.4.1 General description of the dystrophic line

1.4.1.1 Derivation

A strain of chicken exhibiting the genetically transmitted condition of muscular dystrophy was derived by selective breeding of a spontaneous mutation in New Hampshire chickens (Asmundsen & Julian, 1956). The disorder involves a dysfunction of the fast twitch muscles, most noticeable in the inability of the birds to turn over or rise to their feet when placed on their backs. A marked increase in pectoral width occurs in these birds; initial hypertrophy of the affected muscles is followed by later atrophy. The disease is transmitted as an autosomal recessive trait, although it may more properly be called co-dominant since heterozygotes also disply some intermediate characteristics (Wilson et al, 1979). A particular strain of the dystrophic line (No. 304) was selected for early onset of the disease; this strain also achieves a body weight closer than other dystrophic strains to that of the normal line (Asmundsen et al, 1963). Outcrossing of this line (No. 304 x normal New Hampshire) produced the dystrophic line (No. 413) and genetically related normal line (No. 412) which were employed in this study.

1.4.1.2 Functional manifestations

The dystrophic condition in the chicken is a progessive one which increases steadily after the first functional impairment is detected; the birds begin to show an inability to right themselves at 2-3 weeks ex ovo. The flip number (number of times

a bird can right itself out of the number of times tested) and wing apposition (distance between the wings when they are apposed over the vertebrae) are two functional tests used to judge the degree of dystrophy. The disease affects primarily the fast twitch muscles and proceeds in a cranio-caudal direction: the superficial pectoral muscle is the most severely affected; the PLD and wing muscles also become dystrophic. Some more distal muscles in the leg are less affected and only at later ages (1-2 years). The disease is not so debilitating that it causes early mortality, unless due to deprivation if a bird is left unassisted on its back.

1.4.2 Effects on fast twitch muscles

1.4.2.1 Electrophysiology

Dystrophic muscles show a reduced twitch/tetanus ratio and a decreased maximal rate of rise in the twitch tension at 8-16 weeks (Hoekman, 1976). Since neither the capacity for maximal tetanic tension nor the time needed for muscle relaxation are altered, Hoekman suggested that the main functional problem in dystrophic muscle is a decreased ability to shorten rapidly. Membranes of dystrophic fibres, after d30, exhibit electrical hyperexcitability to mechanical stimuli and abnormal repetitive firing. No other abnormalities, such as an inability to conduct impulses or a failure of transmitter release, have been found (Holliday et al, 1965; Warnick et al, 1979; Wilson et al, 1979). The electrophysiological disturbances were suggested as being due to an alteration, pre- and post-junctionally, in calcium conductance.

1.4.2.2 Ultrastructure

Many alterations have been observed in the ultrastructure of dystrophic muscle fibres. Correlated with the overall initial muscle hypertrophy during the first several weeks is a cellular

hypertrophy which is followed by the appearance of a greater than normal number of fibres, especially those of smaller size, as the muscle atrophies (Julian & Asmundsen, 1963; McMurty et al, 1972; Shafiq et al, 1972). Fibre shapes become rounded and irregular; large fibres often split. The number of nuclei increases and they are located peripherally. This is reflected in the increased DNA content of dystrophic fibres (Morgan & Herrmann, 1965). Total fat content of the muscle is elevated from an early age; fat and connective tissue infiltrate dystrophic muscle as the disease progresses (Asmundsen et al, 1956). Phagocytic areas are evidence of the destruction of degenerating fibres (McMurty et al, 1972).

A general proliferation of internal membranes (from mitochondria, sarcoplasmic reticulum and transverse tubules) also occurs (Scales et al, 1977). Beringer (1978) regarded the increase of mitochondria and of fibre splitting as an attempt by the cell to compensate for a deficiency in its capacity for anaerobic metabolism.

1.4.2.3 Metabolism

Ashmore & Doerr (1971) stated that the increased rate of amino acid incorporation in dystrophic fibres implies a rapid rate of growth. This, in turn, would put a higher demand for energy on the metabolic system of the fibre. Such rapid growth is witnessed in the initial stage of muscle hypertrophy. Increased levels of cholesterol, particularly in the sarcoplasmic reticulum, also occur in dystrophic fibres (Hsu & Kaldor, 1971; Stewart et al, 1977). Rodan et al (1974) proposed that the increased lipid content of dystrophic fibre membranes alters their permeability, thus causing the increased levels of adenyl cyclase (a membrane-bound enzyme; EC 4.6.1.1) which they found in dystrophic pectoral muscle at 6 weeks. The plasma membrane has been found to degenerate in dystrophic fibres,

thus indicating, along with the fibre splitting, an abnormal membrane milieu. Changes in membrane structure or composition could allow muscle enzymes to leak into the blood. They may also account for electrophysiological abnormalities which are thought to involve faulty calcium conductance in membranes (see 1.4.2.1).

Morphological alterations appear several weeks after hatching and may reflect compensatory or degenerative changes in response to earlier biochemical abnormalities within the fibre, such as altered enzyme production. Increased levels of muscle enzymes appear in the blood, presumably due to "leaky" membranes, and have been used as markers for dystrophy. Excess plasma aldolase (EC 4.1.2.13) and glutamic oxalic transferase (glutamic oxaloacetic transaminase, EC 2.6.1.1) were the first such biochemical markers noted (Cornelius et al, 1959). Creatine kinase (CPK, EC 2.7.3.2) and AChE are also elevated in the blood several weeks ex ovo (Wilson et al, 1973b). Excess pyruvate kinase (PK, EC 2.7.1.40) and thymidine kinase (EC 2.7.1.75) have also been detected in the plasma of dystrophic chickens (Weinstock & Dju, 1971; Weinstock et al, 1977).

Inside the dystrophic muscle, there is a change in the synthesis of metabolic enzymes: a decrease in enzymes involved in anaerobic metabolism (e.g. phosphorylase) and an increase in those involved in aerobic metabolism (e.g. SDH). This results in staining patterns for metabolic enzymes in dystrophic fibres which resemble those of slow tonic fibres (Ashmore & Doerr, 1971).

1.4.2.4 Changes in AChE and ψChE

In addition to elevated levels in plasma, AChE is also increased in aqueous extracts of dystrophic pectoral muscle 5 weeks ex ovo and later (Wilson et al, 1969 & 1973a). Interpretation of the decontrol of the forms of this enzyme is difficult (as discussed

in 1.1.3). Wilson et al reported the presence in mature dystrophic muscle of an "embryonic" enzyme pattern: substantial activity of the lighter forms of AChE (presumably 4S and 7S) occurs in dystrophic fibres, whereas it disappears in normal, adult fast twitch muscle. They also found high AChE activity in the plasma from dystrophic chickens, like that found in normal embryonic blood, and proposed that such enzyme activity is due to a leakage of AChE from dystrophic muscle. The most prominent AChE form in plasma is that represented by the slowest band, although a few faster bands are also apparent. However, no definite attribution of the subunit size (S value) or structure can be given to these bands.

ψChE levels also increase in affected muscles, but not in the plasma of dystrophic chickens. Although a similar analysis by electrophoresis of the forms of ψChE was not as thorough as that done for AChE, the forms of ψChE in plasma of normal and dystrophic chickens are apparently similar. Most of the activity is in the slowest electrophoretic band, while several faster bands also occur. A significant problem in these studies of the enzyme forms in the chicken is that they employed aqueous extraction which only partially solubilizes the enzyme activity in muscle, particularly failing to extract the 20S form.

The localization of AChE is abnormal in dystrophic muscle. Usually confined to within 50μm of the endplate, histochemical stain for AChE is spread over a wide area (ca 250μm) around the endplate; excess stain appears in the sarcoplasm and around the nuclei of dystrophic fibres (Wilson et al, 1975; Patterson & Wilson, 1976a&b). Histochemical stain for ψChE is also more prominent on the surface membrane of dystrophic fibres around the endplate. Since membrane-bound forms are those least solubilized by Wilson's aqueous extraction method, only a

partial, and possibly misleading, characterization has been done of the changes in the forms of AChE and ψChE in normal and dystrophic chicken muscle.

1.4.3 Dystrophy as an embryonic condition

Wilson et al proposed that dystrophy is due to a lack of the proper suppression of embryonic enzyme forms since he detected the same prevalence of light forms of AChE in both normal embryonic and adult dystrophic muscle. Similar suggestions that dystrophy is due to a retardation of muscle fibre maturation were made by others: Kaplan & Chan (1962; based on a failure of the normal control of LDH isozymes); Cosmos et al (1979; regarding the maturation of the metabolic enzyme systems) and Morgan & Hermann (1965; regarding a slower than normal decrease in DNA levels ex ovo).

Beringer (1978), however, proposed that the increase in the number of fast twitch fibres in dystrophic muscle with enzymes for aerobic metabolism may not be due to a failure in the differentiation of enzymes for anaerobic metabolism. Instead, it may be due to degenerative change of previously differentiated anaerobic, fast twitch fibres back into aerobic, fast twitch fibres. A defect may prevent the proper functioning of the anaerobic enzymes or cause a higher demand for energy which cannot be met by the anaerobic system. Then the dystrophic fibres may revert to synthesizing, in addition, enzymes for aerobic metabolism in an attempt to supply the extra energy required. Ashmore & Doerr (1971) found that, although dystrophic fibres differentiate ex ovo into anaerobic fibres, they also produce high levels of oxidative enzymes. Thus, they perceive the dystrophic process as being more complex than a simple retardation of fibre maturation.

In normal fast twitch fibres the transition to their characteristic adult patterns of metabolic enzymes and myosin is normally completed during the first several weeks ex ovo (see 1.3.2.3). It is not surprising, therefore, that most morphological and biochemical criteria used thus far have not detected signs of dystrophy before the first 1-2 weeks ex ovo.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Sigma Chemicals

Acetylthiocholine iodide (AThChI)

5,5'-dithiobis-2-nitrobenzoic acid (DTNB)

Beef liver catalase (EC 1.11.1.6)

Horse liver alchohol dehydrogenase (ADH, EC 1.1.1.1)

E.coli β-galactosidase (Grade IV, β-D-galactoside galactohydrolase, EC 3.2.1.23)

Ethyleneglycol-bis-(β-amino-ethylether)-N, N'tetraacetic acid (EGTA)

N-ethylmaleimide (NEM)

Benzamidine hydrochloride

Bacitracin

Eserine (1'-methylpyrrolidino (2';3';2;3)1,3-dimethylindolin-5-yl N-methylcarbamate, Physostigmine)

Collagenase (Type VI, Clostridiopeptidase A from *C. histolyticum*, EC 3.4.24.3)

Koch-Light

Butyrylthiocholine iodide (BuThChI)

isoOMPA (tetra*iso*propylpyrophosphoramide)

Rohn-Hass

Triton X-100

U.S.A. - Japan Cooperative Research Program

A gift of: pepstatin

leupeptin

Wellcome Research Laboratories (a gift from Dr. H.F. Hodson)

BW284C51

Rhone-Poulenc

10-(2-diethylaminopropyl)phenothiazine hydrochloride (ethopropazine, Parsidol)

The following drugs were obtained from the companies listed:

Guilini Pharma	:	Prajmaline
Hoffman-La Roche Inc.	:	Methiothepin
Laboratories Beaufort	:	IAPP (N-isopropylamino-2-pyrimidine-phosphate)
Merck, Sharp & Dohme Research Laboratories	:	Cyproheptadine
Sandoz Products Limited	:	Sanomigran
Sigma Chemical Company	:	DPH (Diphenylhydantoin-sodium salt)
E.R. Squibb & Sons, Inc.	:	Cinanserin HCl
Brocades/Great Britain Ltd.	:	Hepzidine

Chickens were obtained from the Imperial College flock of the University of California (Davis) normal (line 412) and dystrophic (line 413) chicken strains.

2.2. Methods

2.2.1 Basic solutions

DTNB reagent:

1mM	DTNB
0.1M	Sodium phosphate buffer pH 7.2

Ellman assay reaction:

1ml	DTNB reagent (containing inhibitor)
0.05ml	AThChI (0.75mM final concentration)
0.02ml	sample

Sucrose gradient buffer:

1M	NaCl
0.1%	Triton X-100
0.01M	Sodium phosphate buffer pH 7.0
and:	
5, 20 or 60%	Sucrose

Extraction buffer:

Basic buffer

1%	Triton X-100
1M	NaCl
10mM	EGTA
0.01M	Sodium phosphate buffer pH 7.0

Complete buffer - basic buffer + protease inhibitors:

2mM	benzamidine
1mg/ml	bacitracin
40µg/ml	leupeptin
20µg/ml	pepstatin
5mM	NEM

2.2.2 Enzyme assays

2.2.2.1 Cholinesterase

ACh is a naturally occurring substrate for AChE and ψChE; but AThCH can also act as a substrate for both these enzymes (as well as BuThCh, for ψChE). When the thiocholine substrates are employed according to the method of Ellman et al (1961), as in Fig. 1B, a thiol is released which combines with DTNB forming a yellow-coloured product, 5-thio-2-nitrobenzoic acid. The change in colour or optical density (ΔOD/minutes, min.) is measured at 412 nm and converted into units of activity (U) by multiplying by factors which take account of the molar absorption coefficient and the extent of sample dilution. The factors employed were: for plasma - ΔOD/min. x 4.2 = U/l; for muscle homogenates (100mg/1.0ml) - ΔOD/min. x 4.2 x 0.011 = U/gram(g) of tissue. One unit of enzyme activity is defined as that amount which hydrolyzes 1μmole of substrate/min. at 25°C under the assay conditions employed.

AThChI (0.75mM) was employed as the substrate when assaying crude tissue samples for both AChE and ψChE activity, since ψChE in the chicken utilizes AThChI as well as BuThChI. AChE activity was determined by employing isoOMPA (10^{-4}M) in the Ellman reaction with a 30-40 min. preincubation period before substrate addition. ψChE activity was determined by employing BW284C51 (5×10^{-5}M) with a 15 min. preincubation period. (See Chapter 3 for determination of effective inhibitor concentrations.)

Enzyme reactions were routinely measured for one minute at 25°C in a LKB reaction rate analyzer (8600) using half the reagent volumes as listed in 2.2.1 in small volume cuvettes. Assays were occasionally measured in a Cecil 262 spectrophotometer, in which case full volumes of all reagents were employed. Pipetting was

normally done with an automatic LKB dilutor which increased reproducibility. When enzyme levels were very low, the sample volume was doubled and the appropriate factors adjusted in calculating units of activity.

2.2.2.2 ADH

Horse liver ADH was used as a 4.8S marker on sucrose gradients. An appropriate amount (usually 0.05-0.1ml) was applied to the gradient and aliquots were taken from the peak fractions near the top of the gradient and assayed at 340nm. The assay procedure was:

0.6 ml pyrophosphate-HCL (0.033M) pH 8.8

0.15ml ethanol (2M)

0.3 ml NAD (0.025M in H_2O)

0.05ml sample

2.2.2.3 β-Galactosidase

β-galactosidase was used as a 16S marker on sucrose gradients. An appropriate amount was applied to the gradient. Peak fractions were assayed at 405nm by combining:

0.6 ml Sodium phosphate buffer (0.15M, pH 7.5)

0.1 ml β-mercaptoethanol (1M in H_2O)

0.2 ml o-nitrophenyl -D-galactopyranoside (0.014M in 0.01M Tris-acetate, pH 7.5/0.01M $MgCl_2$)

0.05ml sample

2.2.3 Tissue preparation

2.2.3.1 Muscle homogenization

Fresh muscle was obtained either from chickens killed by cervical dislocation or by biopsy of ether-anaesthetized chickens. The pectoral muscle was sectioned opposite the midpoint of the sternum on the ventral surface of the muscle, one or two inches towards the centre of the muscle in developed birds. Other types of muscles were

sectioned in the middle. In small ALD and PLD muscles a strip was taken across the entire medial section. If blood appeared on the tissue it was removed by blotting on a dry tissue. Whole muscles were usually employed in embryonic studies.

Muscles and homogenates were kept on ice. Samples (100mg) were homogenized, as soon as possible, in 1.0ml of ice-cold extraction buffer with 20 strokes of a glass-glass hand homogenizer. Part of the buffer was reserved to rinse the homogenizer. All of the protease inhibitors (see 2.2.1), except NEM, were dissolved in the basic extraction buffer immediately before homogenization. Homogenates were centrifuged for 30 min. at 27,000 x g at $4^{o}C$ in a Sorval RC5 centrifuge. The supernatant was reserved and assayed. The extent of enzyme solubilization was 90-100% (for eight birds tested).

An aliquot of the supernatant was mixed with the protease inhibitor/extraction buffer to which 20mM NEM had been added (3 parts extract/1 part NEM buffer). It was necessary to assay homogenates in the absence of NEM since its presence lowered the amount of enzyme activity detected, presumably by alkylating the reduced DTNB. NEM was important in sucrose gradient analysis of the enzyme forms. First, it protected the molecular forms of the enzymes from degradation (Silman et al, 1978). Second, NEM reduced the background on gradients, presumably by alkylating thiol groups on extraneous proteins in the homogenate, although not affecting the assay of gradient fractions.

2.2.3.2 Perfusion

In certain instances whole body perfusion of chickens was employed to remove blood from the muscles. Saline (0.85%) was introduced into the jugular vein and circulated with a peristaltic pump for ca 20 min. until the fluid removed from the femoral artery became clear and muscles lost their red colour. Chickens were anaesthetized during this procedure.

2.2.3.3 Collagenase treatment

Muscle homogenates (without NEM) were incubated for 1 hour at 37°C with collagenase (250µg/ml, 5 mM $CaCl_2$). The homogenate was centrifuged for 3 min. at 10,000 rpm in a Gelman Hawksly table top centrifuge at room temperature. The supernatant was reserved and mixed with one third its volume of complete extraction buffer with NEM.

2.2.3.4 Plasma

Plasma was obtained from heparinized blood which was extracted from the jugular vein without anaesthetic. Blood cells were pelleted by centrifugation at 10,000 g for 3 min. in a Gelman Hawksly centrifuge and they were discarded. Plasma was assayed without dilution. Before being applied to sucrose gradients, plasma samples were mixed with complete extraction buffer with NEM (3 parts plasma/1 part buffer).

2.2.4 Sucrose gradient analysis

Five to twenty percent sucrose gradients were prepared mechanically with a Buchler Densi-flo apparatus on top of a 0.5ml 60% sucrose "cushion" in 12ml polyallomer tubes for a Beckman SW40Ti rotor. Samples were applied in appropriate volumes (not exceeding 0.3ml) to give absorbance changes in the peak fractions of the gradient in the range of 0.5 - 1.5 OD after 1-4 hours of incubation. Gradients were centrifuged for 17 hours at 40,000 rev/min. at 4°C in a Beckman L5-65 centrifuge. Approximately 65 fractions were collected from each gradient, with a Densi-flo apparatus, starting at the top. The markers used for gradient calibration were ADH, β-galactosidase and beef liver catalase. Assays for the former

two enzymes are given in 2.2.2. Fractions with the catalase marker (11.4S) were diluted with 1.0ml of water and the absorbance was read at 405nm.

Gradient fractions were developed for AChE activity by preincubating for 30min. in 0.5ml of (double concentration) DTNB reagent with isoOMPA before the addition of 0.5ml substrate (1.5mM). Gradient fractions were developed for ψChE activity by incubating them with 0.75mM BuThChI and 10^{-5}M BW284C51 in DTNB buffer.

Most gradient profiles shown represent a pool of samples from at least four chickens. The area under each curve was determined using a Hewlett Packard Digitizer (Model 9864A) and is expressed as a percentage of the total activity on the gradient. The contribution of each molecular form was determined by averaging the percentages found thus for at least four birds of each age, unless stated otherwise. The activities of ψChE in the L_1 and L_2 forms often were not clearly distinguished from each other and, therefore, are sometimes indicated as $L_1 + L_2$. Incubation factors (IF) are indicated on certain gradient profiles to enable a relative comparison of the activity detected on gradients of different samples. IF was derived:

$$\text{mg of tissue on gradient} \times \text{hours of incubation} \times \frac{1}{6.8}.$$

An IF of 1, for example, represents a gradient with 6.8mg of tissue (usually 0.10ml) which was incubated for one hour.

CHAPTER 3

CONDITIONS AFFECTING THE ENZYME ASSAYS

3.1 Effective Inhibitor Concentration

3.1.1 IsoOMPA

The levels and ratios of AChE and ψChE vary considerably in tissues of the chicken depending on the derivation of the tissue (e.g. plasma/muscle, normal/dystrophic). In order to determine accurately the level of either AChE or ψChE it is necessary to ensure complete inhibition of the other cholinesterase with a specific inhibitor. The effectiveness of isoOMPA in inhibiting ψChE was tested using a plasma sample which contained ca 90% ψChE activity and AThChI (0.75mM) as substrate (Fig.4A). A concentration of 10^{-4}M is sufficient to inhibit all ψChE activity and was employed in this study. Although isoOMPA is an irreversible inhibitor, a preincubation period is necessary for complete ψChE inhibition; 30-40 minutes (min.) was routinely used in this study.

3.1.2 BW284C51

The reversible AChE inhibitor BW284C51 is generally considered to be effective at a concentration of 10^{-5}M (Silver, 1974). The optimal concentration needed to inhibit chick AChE was examined using a homogenate of normal muscle, containing predominantly AChE, and AThChI (0.75mM) as substrate (Fig.4B). AChE activity in this case is not completely inhibited at 10^{-5}M. Direct comparisons of the enzyme activities in several samples using BW284C51 at 10^{-5}M and $5x10^{-5}$M showed that slightly more AChE activity was inhibited at $5x10^{-5}$M. Since AChE can be quite high in relation to ψChE in certain tissues of the chicken, the most practical inhibitor concentration was $5x10^{-5}$M, not 10^{-4}M, because the amount of available inhibitor was limited. A preincubation period of 15-20 min. was also routinely employed to allow complete inhibition of AChE.

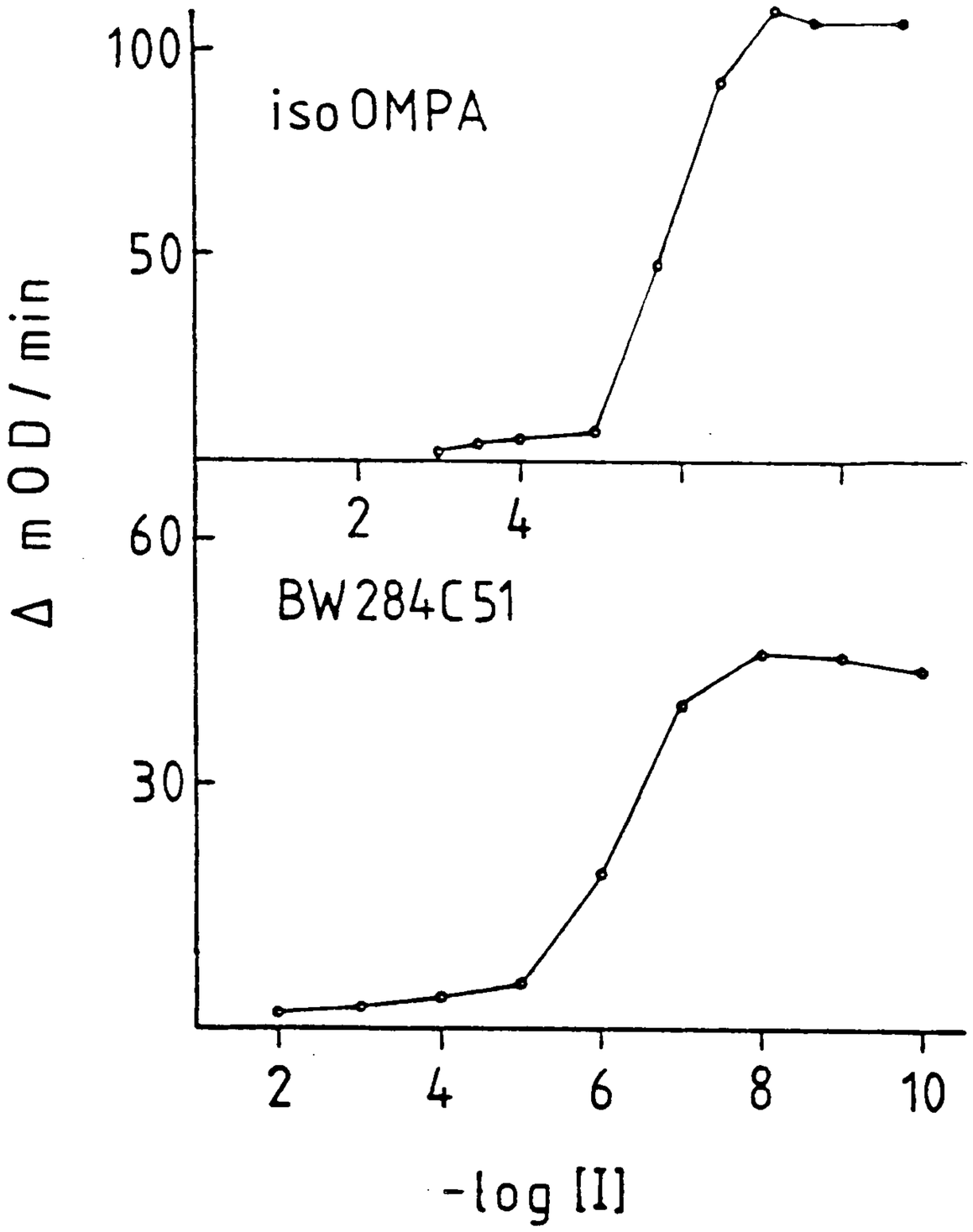


Figure 4. Inhibition of ψChE and AChE by, respectively, isoOMPA and BW284C51.

Varying concentrations of (A) isoOMPA and (B) BW284C51 were employed with AThChI as the substrate. The enzyme source was: (A) plasma and (B) normal pectoral muscle.

When developing sucrose gradient fractions for ψChE activity, BuThChI (0.75mM) was substituted as the substrate. BW284C51 was employed in this case at 10^{-5}M with no preincubation period (since BuCh is not hydrolyzed very rapidly by AChE in the chicken; Blaber & Cuthbert, 1962).

3.1.3 Comparison of the efficacy of isoOMPA versus ethopropazine

Ethopropazine has been employed by other workers (Di Giamberardino & Couraud, 1978; Vigny et al, 1978) at 10^{-4}M to inhibit ψChE in tissues of the chicken and rat. Blaber & Cuthbert (1962), however, reported that 10^{-4}M ethopropazine only partially inhibited ψChE activity in chick plasma. Therefore, the efficacy of this inhibitor was tested against that of isoOMPA (both at 10^{-4}M), using a sample of chick plasma (mostly ψChE) and varying the amount of enzyme which was assayed (Table 2A). Ethopropazine was found not to inhibit ψChE as well as isoOMPA at higher levels of enzyme activity. Since a routine procedure using constant sample volumes and preincubation times was required in this study, isoOMPA was chosen as the more useful ψChE inhibitor.

3.2 Non-specific Esterase Activity

Eserine is an inhibitor of specific cholinesterases (AChE and ψChE) which is useful in detecting non-specific esterase activity. Since ψChE levels are extremely low in certain normal tissues, it is important to confirm that the minimal enzyme activity detected is not due to non-specific esterases which utilize AThChI or BuThChI very slowly. Eserine (10^{-4}M, 50 or 120 min. preincubation) was employed in the assay of AChE and ψChE in a sample of dystrophic pectoral muscle (Table 2B). ψChE was also assayed using BuThChI (0.75mM). AChE and ψChE account for all the enzyme activity hydrolyzing AThChI as substrate. No non-specific esterase activity

TABLE 2

ESTERASE ACTIVITY IN THE CHICKEN IN THE

PRESENCE OF VARIOUS INHIBITORS

A. A comparison of the effectiveness of isoOMPA vs. ethopropazine

Type of enzyme activity (inhibitor[a])	Activity[b] (ΔmOD/min) Sample volume assayed in 1.0ml: 0.01ml	0.02ml	0.05ml
Total esterase (no inhibitor)	54		
DTNB/substrate blank	1		
No ChE (isoOMPA+BW284C51)	2		
ψChE (BW284C51)	54		
AChE			
(isoOMPA)	9	15	46
(ethopropazine)	9	25	67

B. Nonspecific esterase activity in the dystrophic pectoral muscle

Type of enzyme activity (inhibitor[a])	Activity[b] (ΔOD/min/ml)
Total esterase	140
AChE (isoOMPA)	121
ψChE (BW284C51)	24
Nonspecific esterase (isoOMPA+BW284C51)	3
Nonspecific esterase (Eserine)	
AThChI substrate	
50 min. preincubation	5
120 min. preincubation	3
BuThChI substrate	3

[a]Inhibitors used are explained in the text (3.2). Concentrations employed were: isoOMPA (10^{-4} M); BW284C51 (5×10^{-5} M); ethopropazine (10^{-4} M); eserine (10^{-4} M). Normal plasma was assayed in (A).

[b]Values represent the mean of 2 assays

was detected in chick muscle. A sucrose gradient of a muscle sample was also developed with BuThChI and eserine. No peaks of activity appeared even after a prolonged (2 day) reaction time.

3.3 The Effects of NaCl and Sucrose

3.3.1 NaCl

Tissue extraction and sucrose gradient sedimentation were done in the presence of 1M NaCl. The concentrations of NaCl present during the enzyme assays of crude sample or gradient fractions were, respectively, 0.02M and 0.2M. The effects of such NaCl concentrations on enzyme assays were examined (Table 3A) and found not to alter, substantially, the activity of either AChE or ψChE. Even prolonged storage in 1M NaCl at 4^{o}C did not alter enzyme activity.

3.3.2 Sucrose

Sucrose gradient fractions were assayed for enzyme activity in the presence of 1-4% sucrose gradient buffer. The effect of this range of sucrose concentrations (with varying levels of NaCl) is to decrease AChE and ψChE activity slightly more than NaCl alone (Table 3B); but this effect does not increase in a direct proportion with increasing sucrose concentrations. Control blanks were developed in parallel with gradient fractions using water and sucrose (5,20 and 60%). Regardless of the length of the reaction time, the absorbance of sucrose blanks always differs from the water blank: 5% sucrose reduces the OD as compared to water, whereas 20% and 60% sucrose result in higher OD. This effect is nonlinear along the gradient - the OD of the blanks increases between fractions 10-20 (out of a total ca 65 fractions) and reaches a plateau which is maintained to the bottom of the gradient. Sucrose, itself, alters

TABLE 3

THE EFFECTS OF VARIOUS REAGENTS ON

AChE AND ψChE ACTIVITY

	AChE		ψChE	
	(ΔOD/min./ml)	% of control	(ΔOD/min./ml)	% of control
A. NaCl				
Assay concentration				
0.0 M	142		93	
0.02	130	92	91	98
0.04	134	94	93	100
0.10	142	100	94	101
0.20	123	87	88	94
Storage of enzyme (20h) in NaCl				
0.0 M	63		34	
1.0	67	106	39	115
B. Sucrose (+NaCl)				
Assay concentration				
0% (0.00 M)	142		93	
1 (0.03)	110	77	91	94
2 (0.06)	122	86	75	81
4 (0.12)	119	83	73	78
C. ATP (10^{-3} M)				
Change with time				
0h	47		13	
43h @ 4°C	48	102	15	115
43h @ Rm. temp.	44	106	13	100
With NaF (at 0 h)				
0 M	49		9	
0.1	9	17	8	89

A sample of plasma from a dystrophic chicken was employed in (A) and (B). A homogenate of dystrophic pectoral muscle was employed in (C) (see 4.2.2.1 for discussion).

the colour of DTNB solution in an inexplicable way; this effect increases with time. The general effect of sucrose on optical density was taken into consideration when determining the baselines of gradient profiles.

In order to reduce the effect of sucrose in gradient analysis, the reaction time was kept as short as was practical (ideally 1-2 hours). Gradients of normal muscle samples, however, routinely required longer reaction times; ψChE gradients needed the longest incubation times (1-4 days).

CHAPTER 4

FAST TWITCH MUSCLES

4.1 General Introduction

4.1.1 Characterization of the molecular forms of AChE and ψChE in muscle

4.1.1.1 Sedimentation coefficients

AChE and ψChE in muscles of the chicken exist in various molecular forms as seen in their sedimentation coefficients (S) which were determined by sucrose density gradient velocity sedimentation (Table 4). The sizes of AChE forms agree very closely with those in chick muscle as reported by others since this study began (Bon et al, 1979; Rotundo & Fambrough, 1979): 4.7, 6.6, 11.1, 14.7 and 19.9S. The series of ψChE forms, not previously reported in the chicken, is very similar to the AChE series, except that each form is 0.5-1.0S smaller than the corresponding AChE form: 4.0, 5.9, 10.5, 14.1 and 19.2S.

The two light (L_1,4S; L_2,6S) and one medium (M, 11S) forms are considered to correspond to the monomer (L_1), dimer (L_2), and tetramer (M); whereas the heavy (H) forms of ca 15S (H_1) and 20S (H_2) correspond to tailed structures with several tetrameric heads (see 1.1.3).

4.1.1.2 Collagenase treatment

The asymmetric forms of AChE from Electrophorus have been shown to contain significant amounts of hydroxyproline, hydroxylysine and glycine which are indicative of a collagenous component (Bon et al,1976; Rosenberry & Richardson, 1977; Anglister & Silman, 1978). These amino acids are not similarly found in large amounts in the 11S tetramer. Furthermore, treatment of the asymmetric forms with collagenase removes a major portion of these residues while not substantially altering the percentages of the other amino acids (Anglister & Silman, 1978). Collagenase treatment of asymmetric forms also produces faster sedimenting molecules due to their decreased asymmetry following the removal of a part of the collagen tail (Bon & Massoulié, 1978).

TABLE 4

SEDIMENTATION COEFFICIENTS OF THE MOLECULAR FORMS OF AChE AND ψChE IN MUSCLES OF THE CHICKEN

	L_1	L_2	M	H_1	H_2
AChE	4.7	6.6	11.1	14.7	19.9
S.E.M.	±0.04	±0.07	±0.06	±0.18	±0.11
n	8	8	8	6	8
ψChE	4.0	5.9	10.5	14.1	19.2
S.E.M.	±0.10	±0.07	±0.11	±0.14	±0.24
n	10	9	10	6	5

Values represent the mean sedimentation coefficient ($s_{20,w}$) in Svedberg units and the S.E. for n determinations. The values of $s_{20,w}$ for horse liver alcohol dehydrogenase (4.8S), beef liver catalase (11.4S), and E.coli β-galactosidase (16.0S) and the positions of these standards on 5-20% sucrose gradients were used in determining the sedimentation coefficients reported.

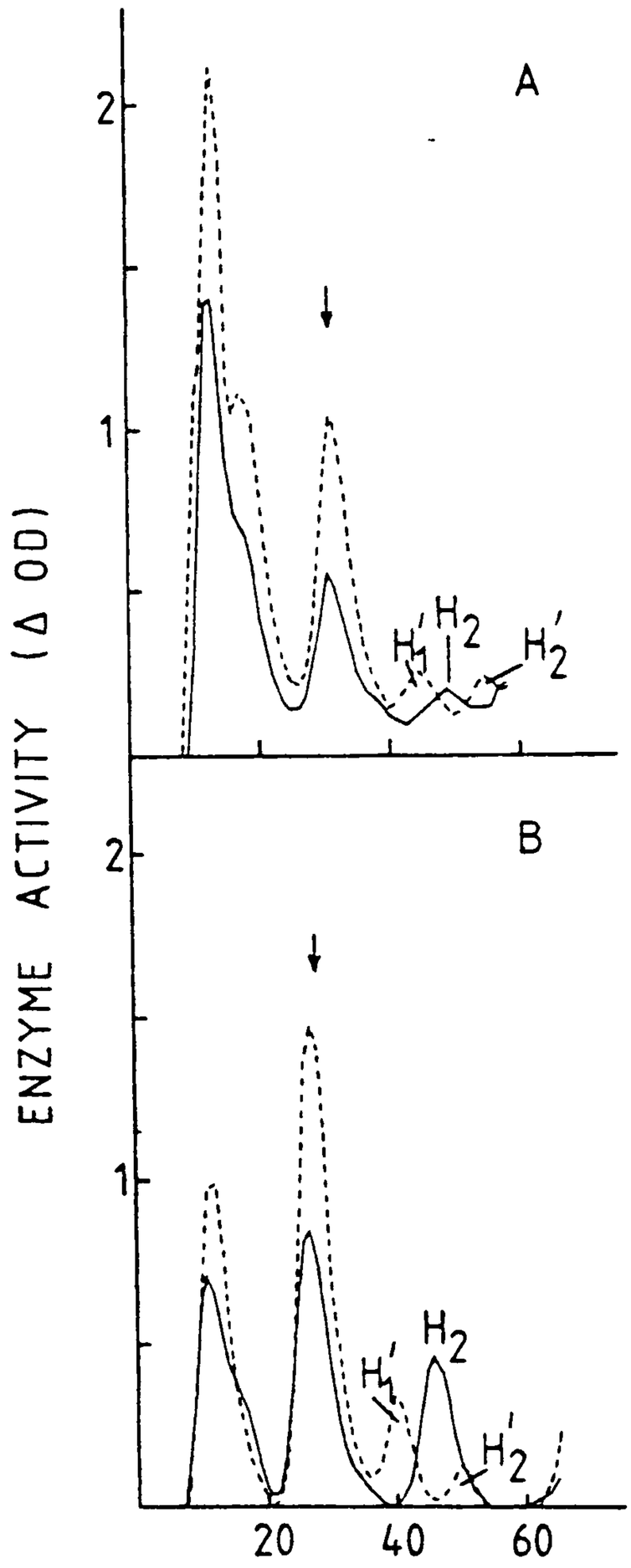


Figure 5. The effect of collagenase on the molecular forms of (A) AChE and (B) ψ ChE.

Control homogenates (——) were incubated at 37°C with 5mM $CaCl_2$; collagenase treated homogenates (- - -) were incubated similarly as described in 2.2.3.3

H_2 is the largest native enzyme molecular form; H_2' is derived from H_2 by collagenase treatment. H_1 is not clearly distinguished on control curves because it is hidden as a shoulder in the M peaks; however, H_1' is clearly distinguished from M following collagenase treatment. H_2 was determined on these gradients to sediment at: (A) 18.0S; (B) 18.7S. (These values are smaller than usual, possibly due to non-linearity at the bottom of the gradient.) H_1' and H_2' were, respectively: (A) 16.5S and 20.5S; (B) 16.3S and 20.3S.

The following details apply to this and all subsequent figures of sucrose gradient profiles. Five to twenty per cent sucrose gradients were prepared and analyzed as described in 2.2.4; the activity of each fraction was read at 412nm. The left margin of the profile corresponds to the top of the gradient. The arrows indicate the position of the catalase (11.4S) marker.

When preparations of AChE from chicken muscle are treated similarly with collagenase (see 2.2.3 for method), only the faster two forms are altered (Fig. 5). H_1 and H_2 become faster sedimenting, presumably because they also become less asymmetric when a portion of the tail is lost. This proves that there are two tailed forms of AChE in the chicken. No smaller tailed form corresponding to 8S in Electrophorus (i.e. one tetramer on a tail) has been identified in the chicken. This could be because such a form (possibly ca 9S) would sediment too closely to the 11S peak to be distinguished.

The same structural model for ψChE has been presumed, by analogy, due to the similar series of sizes of its molecular forms. It is shown here that collagenase affects the larger ψChE forms in the same way that it affects tailed AChE forms, thus proving that ψChE has a collagenous component. It is, therefore, likely that ψChE exists as collagen-tailed forms and that the structural models for AChE and ψChE are similar.

4.1.2 Factors in enzyme extraction from muscle

Since the aim of this study is to quantitatively investigate the molecular forms of cholinesterases in various tissues, it is necessary to ensure two criteria regarding the enzyme preparations.

4.1.2.1 Extraction

First, the extraction procedure should remove the enzymes as completely as possible from tissue. Early studies of cholinesterases in chicken muscle which employed aqueous or low ionic strength buffer extraction selectively removed only a fraction (the more soluble portion) of the total cholinesterase activities from muscle (see 1.1.3). The buffer employed in this study contained a high concentration of NaCl and Triton X-100 and was found to extract 90-100% of the enzyme

activity, as also shown by others: Dudai & Silman (1974a), Rieger & Vigny (1976) and Rotundo & Fambrough (1979).

4.1.2.2 Preservation of native enzyme forms

A second factor concerning enzyme preparations is that the relative proportion of enzyme molecular forms in the extract should reflect, as closely as possible, their native proportions in the tissue. This involves two factors.

1) All forms should be solubilized to the same extent. Triton X-100 and NaCl must be used in order to extract the heavier forms of AChE. The light forms (4S and 6S) are easily extracted in water and, thus, were the main ones detected in previous electrophoretic studies of AChE in chicken muscle. Since the procedure employed in this study provided for almost complete enzyme extraction, the H forms were apparently solubilized to the same extent as the lighter forms.

2) Once extracted, the various enzyme forms must not be degraded or otherwise altered. Although AChE activity is widely reported to be quite stable at $4^{o}C$ or $-20^{o}C$, the different forms might be interconverted upon storage. This was examined by comparing the forms of AChE and ψChE in fresh muscle with those in frozen muscle, stored either as intact tissue or as a homogenate (Fig. 6). More degradation of larger forms into smaller ones occurs for both enzymes when muscle is stored intact rather than as a homogenate. This may be due to the longer period of activity of proteases in the interior of the intact tissue than in the homogenate where enzyme and proteases were diluted in an anti-protease media (see 2.2.1) before homogenization and freezing. Such an anti-protease medium has been shown to be effective in reducing degradation of fresh enzyme in chicken muscle (Silman et al, 1978). The physical effects of freezing may also be greater on enzyme which is restricted to the membrane than on enzyme which is solubilized.

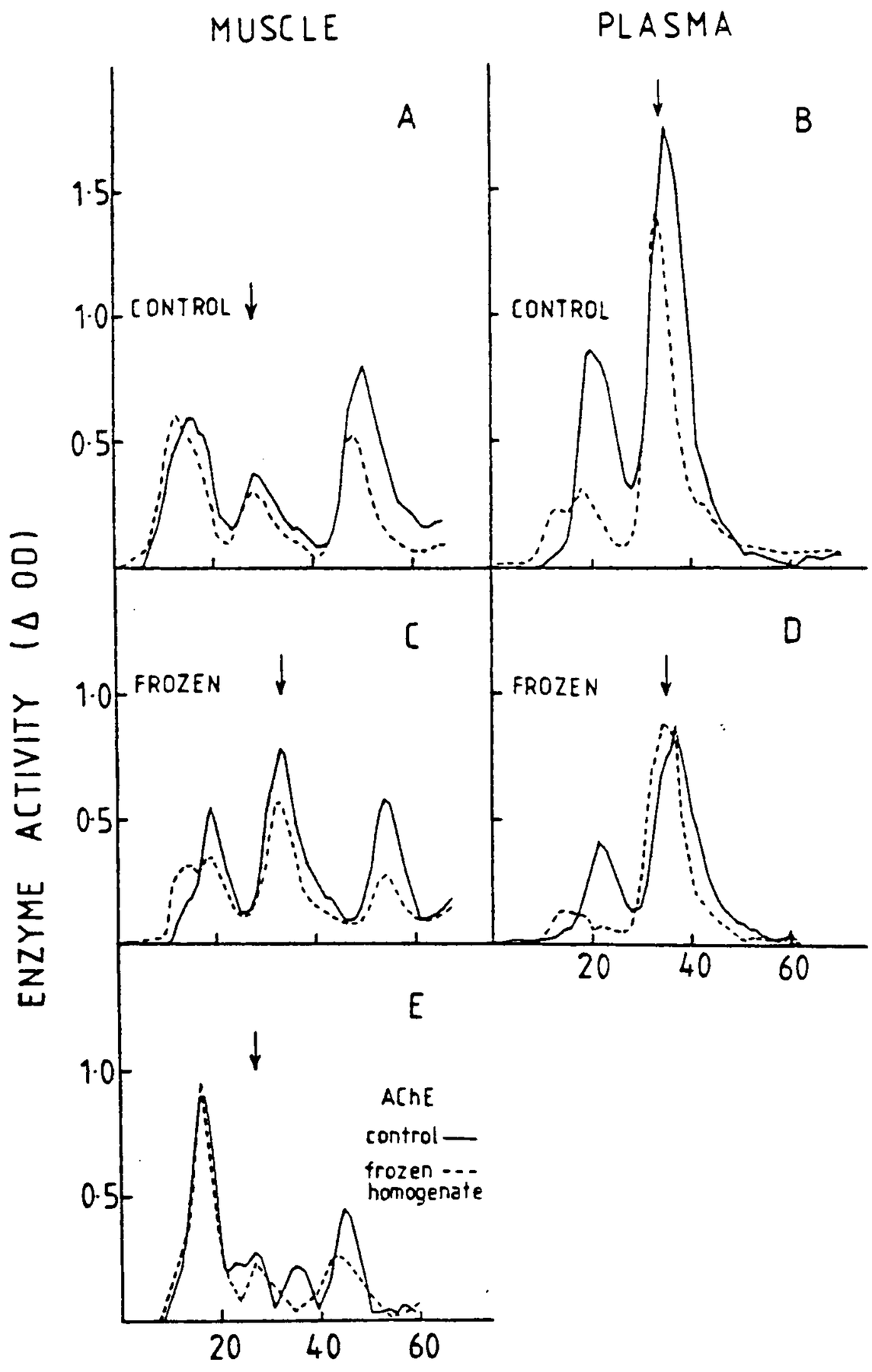


Figure 6. The effects of freezing on the molecular forms of AChE and ψ ChE.

In A-D, AChE (———), ψChE (- - -). (A), fresh muscle homogenate. (B), fresh plasma. (C), frozen muscle, homogenized after 30 days storage at -20°C. (D) plasma frozen 11 days at -20°C. (E) AChE in muscle: fresh (———) or after being frozen as a homogenate with protease inhibitors for 11 days at -20°C (- - -).

The tetrameric forms of both AChE and ψChE appear to be quite stable. Degradation of both AChE and ψChE in frozen, intact muscle results in the conversion of H_2 activity into the M form. The extent of H_2 degradation is reduced when tissue is stored frozen as a homogenate, while the H_1 (ca 15S) form disappears under both storage conditions. The globular AChE forms, especially M, in plasma and in muscle homogenates with antiprotease medium withstand freezing well. Vigny et al (1979a) similarly found that the largest tailed AChE form in the rat (16S) was easily degraded to the globular tetramer by proteolytic treatment with trypsin. The M form, however, was relatively resistent to degradation either by proteolysis or by reduction, while the dimer (L_2) was easily dissociated into monomers (L_1) by proteolysis and reduction with dithioerythritol. In this study of ψChE degradation, a particular loss of the two light forms in frozen muscle and of L_2 and M in frozen plasma indicates that ψChE, generally, may be more labile than AChE. These results suggest that, in both AChE and ψChE, the connections between tetramers and tail may be more labile, while the inter-subunit bonds within the tetramers may be relatively more stable. ψChE, particularly the L forms, also appears to be more labile than AChE.

4.1.2.3 Summary

The procedures used in this study resulted in maximal enzyme extraction from muscle and minimal degradation due to the action of proteases. Fresh (unfrozen) homogenates were always employed in the assay of enzyme activity or in the analysis of forms by sucrose gradient centrifugation. Although the homogenization procedure itself may cause some degradation by physical disruption of the molecules, as many precautions as possible were undertaken in order

to extract the molecular forms of AChE and ψChE in a similar proportion as they occur in situ.

4.2 Pectoral Muscle

4.2.1 AChE

4.2.1.1 Results

Enzyme Levels

AChE activity increases in embryonic pectoral muscle during the last 7 days in ovo (Fig. 7; Tables 5 and 6). Immediately after hatching, AChE activity decreases in normal muscle and is maintained at very low levels after d30 (Tables6 and 7). The levels of AChE in dystrophic pectoral muscle show a similar pattern during development in ovo, except that they are lower than normal; this difference is most obvious at the peak of AChE concentration in ovo on d19. The decrease in AChE concentration ex ovo is slower during the first 30 days in dystrophic muscle. A later peak of enzyme activity occurs around d60, with levels decreasing progressively thereafter.

When the total amount of enzyme in the muscle is considered (Fig. 8A), it is apparent that the amount of AChE in normal muscle is relatively constant during muscle maturation ex ovo. The sudden decrease in enzyme concentration immediately after the time of hatching is due to a dilution of the enzyme by the rapidly increasing muscle mass, rather than being a real decrease in total enzyme activity. The total amount of AChE in dystrophic muscle, however, increases continually from d4 ex ovo, thus causing the more gradual decline in AChE concentration during the initial period of rapid muscle growth.

In very old (d600) dystrophic pectoral muscle AChE concentrations approach normal levels (ca 5X normal). However, the

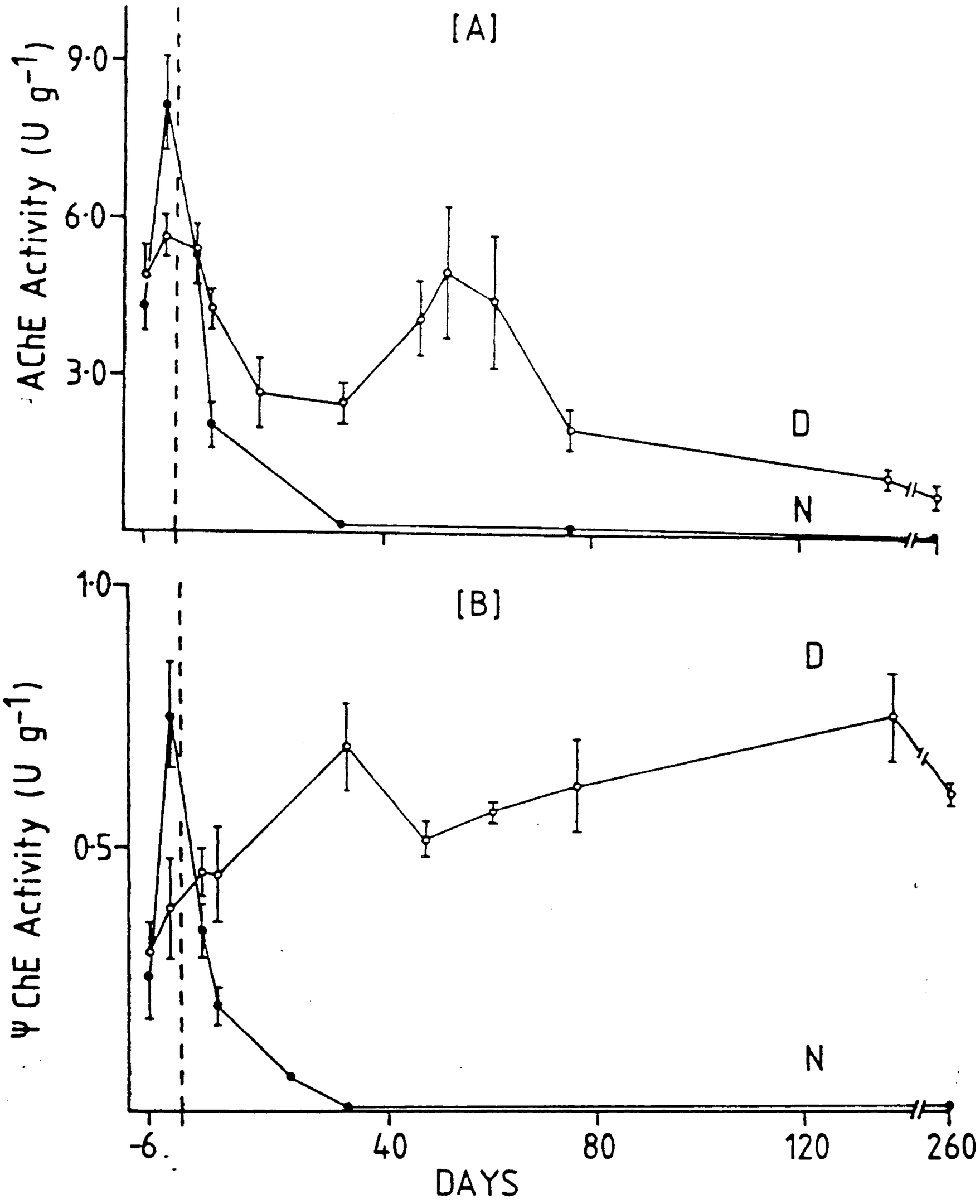


Figure 7. Changes in (A) AChE and (B) ψChE concentrations in developing normal (●——●) and dystrophic (o——o) pectoral muscles of the chicken.

The dashed line represents the day of hatching in this and subsequent graphs. Each point is the mean of 3-8 values; bars represent the standard error of the mean (S.E.M.).

TABLE 5

THE CHANGING FORMS OF AChE IN DEVELOPING NORMAL PECTORAL MUSCLE

Days	% 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

TABLE 6

CHANGES IN THE LEVELS OF L_2 AND H_2 AChE IN DEVELOPING NORMAL AND DYSTROPHIC CHICK PECTORAL MUSCLE

		Day 14 in ovo	Day 19 in ovo
L_2			
Concentration (U/g)	N	2.65	1.01
	D	2.55	0.90
% of total AChE activity	N	42	14
	D	41	16
Total (U/muscle)	N	0.468	0.296
	D	0.384	0.263
% change from normal (total L_2)		-18	-11
H_2			
Concentration (U/g)	N	0.828	3.95
	D	0.776	2.99
% of total AChE activity	N	19	55
	D	19	53
Total (U/muscle)	N	0.212	1.16
	D	0.178	0.87
% change from normal (total H_2)		-16	-25

The terms L_2 and H_2 are defined in Table 4. N = normal; D = dystrophic.

[a] Only values for dystrophic muscle were available for day 60.

TABLE 6 -- Continued

Day 4	Day 7	Day 32	Day 60[a]
0.95	0.21	0.025	
1.91	1.42	1.05	1.33
21	14	18	
37	36	51	41
0.399	0.25	0.40	
0.876	2.04	27.5	116
+120	+723	+6800	
1.77	0.62	0.072	
1.44	1.11	0.433	1.01
39	42	52	
28	28	21	31
0.74	0.74	1.16	
0.69	1.59	11.3	88
-7	+113	+879	

TABLE 7

LEVELS OF AChE AND ψChE IN OLDER NORMAL AND DYSTROPHIC PECTORAL MUSCLE

Age (months)		AChE (U/g)[a]		ψChE (U/g)[a]	
		Normal	Dystrophic	Normal	Dystrophic
12			0.170		0.027
	S.E.M.		±0.073		±0.002
	n		2		2
19		0.059	0.303	0.023	0.243
	S.E.M.	±0.006	±0.105	±0.016	±0.110
	n	2	2	2	2

[a]Activity is expressed as the mean of n birds tested ± the standard error of the mean (S.E.M.).

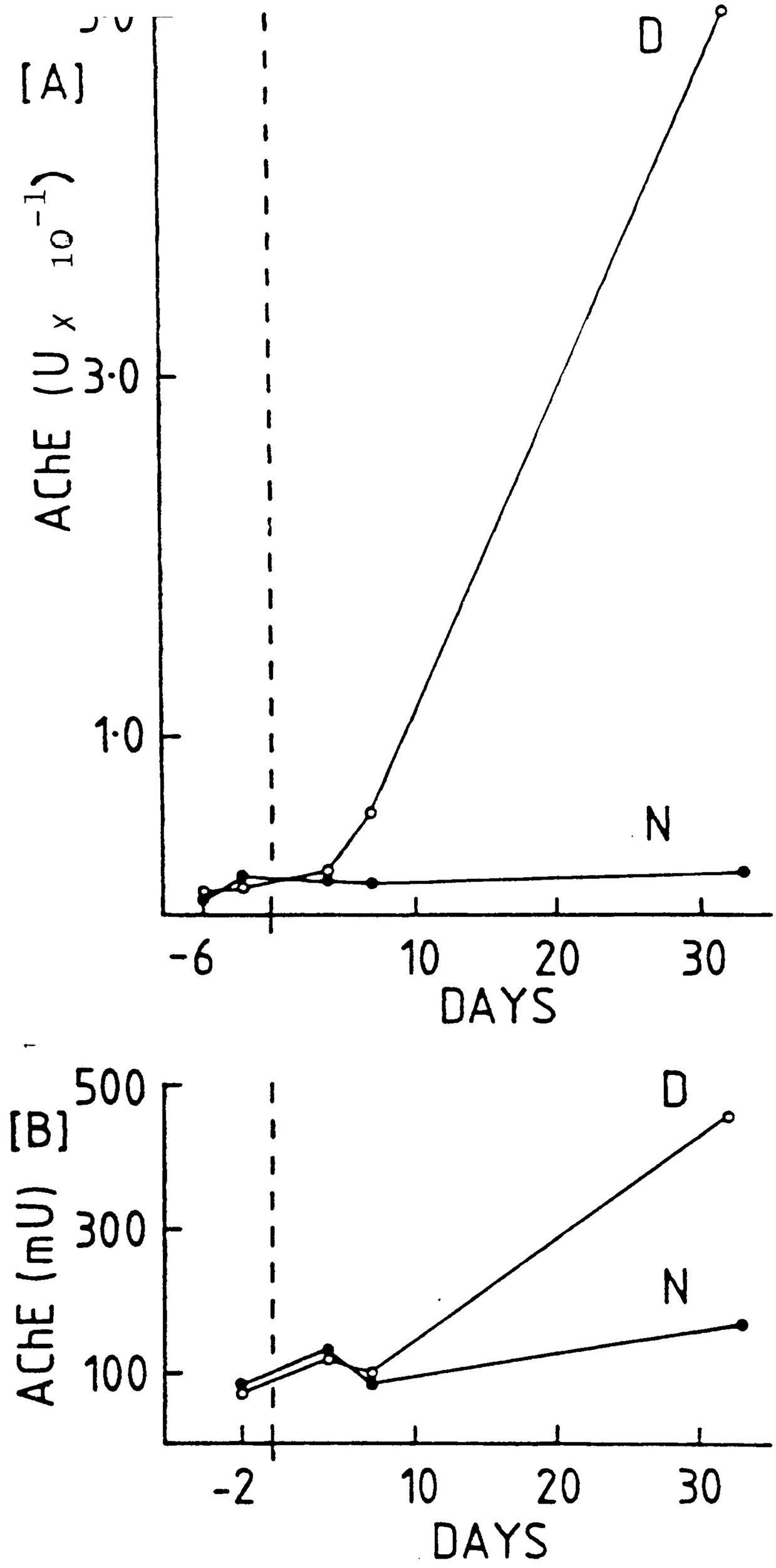


Figure 8. Changes in total AChE in developing normal (●——●) and dystrophic (o——o) muscles in the chicken. (A) Pectoral muscle; (B) PLD muscle.

Each point represents the mean amount of enzyme activity in the total muscle per bird.

proportion of the muscle mass which is due to muscle fibre at d600 is only ca 10%, as compared to ca 90% in normal muscle (information to be published in a thesis by Mr. J. Pizzey from this laboratory, Biochemistry Department, Imperial College). Therefore, the actual increase in AChE concentration per gram muscle fibre is ca 45X normal even in the very old dystrophic muscle.

Enzyme forms

The molecular forms of AChE in embryonic pectoral muscle change progressively from being predominantly L_2 at younger ages to predominantly H_2 just prior to hatching (Fig.9; Tables 5 and 6). The proportion of each form is similar in normal and dystrophic muscle at d14 and d19 in ovo. The H_2 form is maintained as the major form in normal muscle throughout further development ex ovo. In dystrophic muscle, however, there are abnormally high levels of L_2 AChE from d4 and throughout later maturation. This abnormal enzyme regulation involves a selective overproduction, first, of the L_2 form. Later (e.g. by d7) this causes increased levels of the larger forms, as well. The level of L_2 AChE remains disproportionately high in older dystrophic muscle, until H_2 again becomes the predominant form in very old muscle (Fig. 10 and 11).

4.2.1.2 Discussion

Normal pectoral muscle

The changing proportions of AChE forms as detected in this study, along with the previously reported histochemical localization of AChE in developing embryonic muscle (see 1.1.4), substantiate the idea that H_2 is the endplate-specific form of AChE in fast twitch muscles of the chicken. The light forms (L_1 and L_2) are more soluble and occur in the cytoplasm of myotubes grown in tissue culture (Wilson & Walker, 1974). In embryonic myoblasts and myotubes which

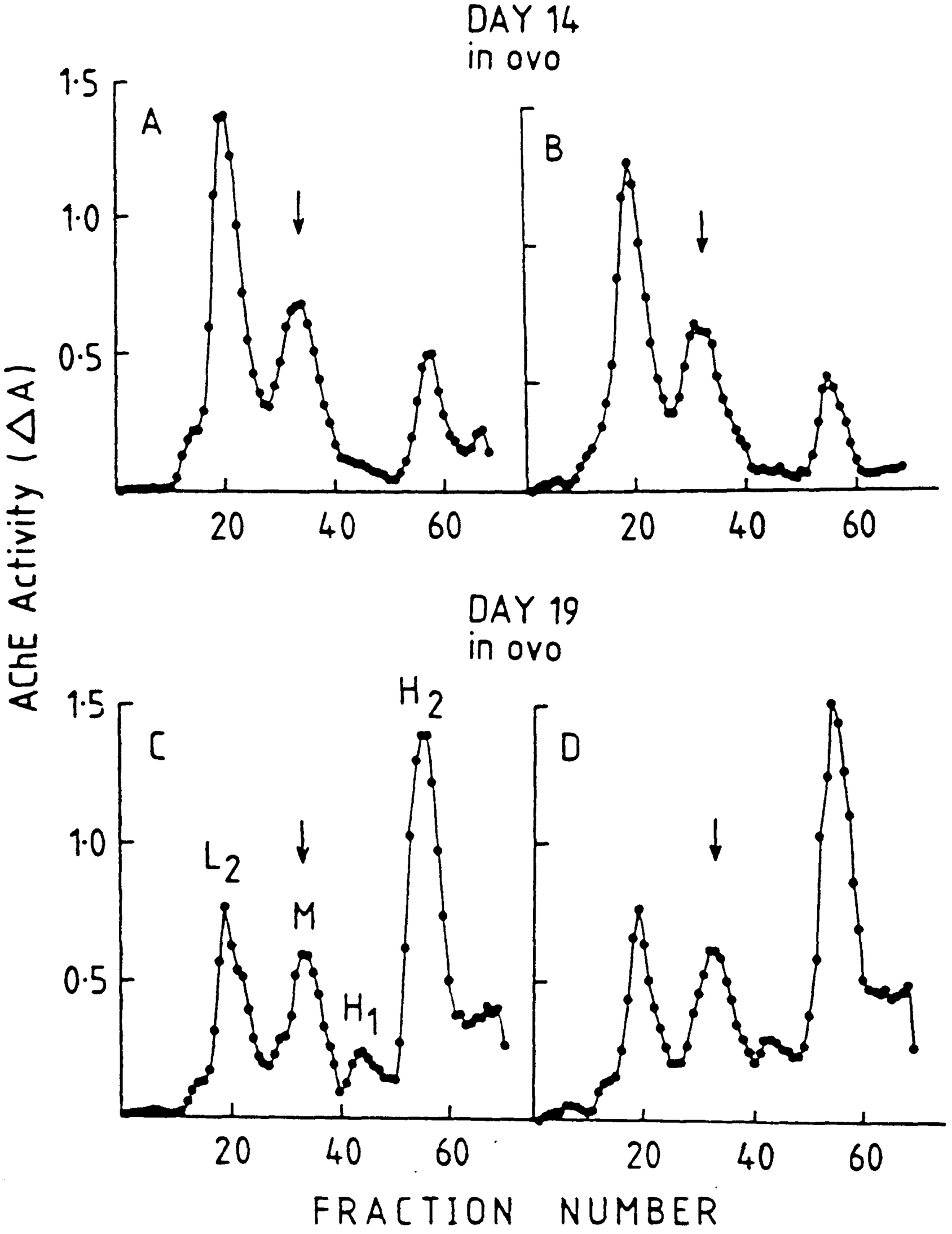


Figure 9. Changes in the molecular forms of AChE during embryonic development of chick pectoral muscle.

Sucrose gradient profiles of enzyme forms in normal (A,C) and dystrophic (B,D) muscle are shown for days 14 and 19 in ovo. IF: A, 1.0; B, 0.75; C, 1.0; D, 1.0. All gradient profiles here and in subsequent figures are representative of at least 4 different birds unless specified otherwise.

Abbreviations above certain peaks are defined in Table 4.

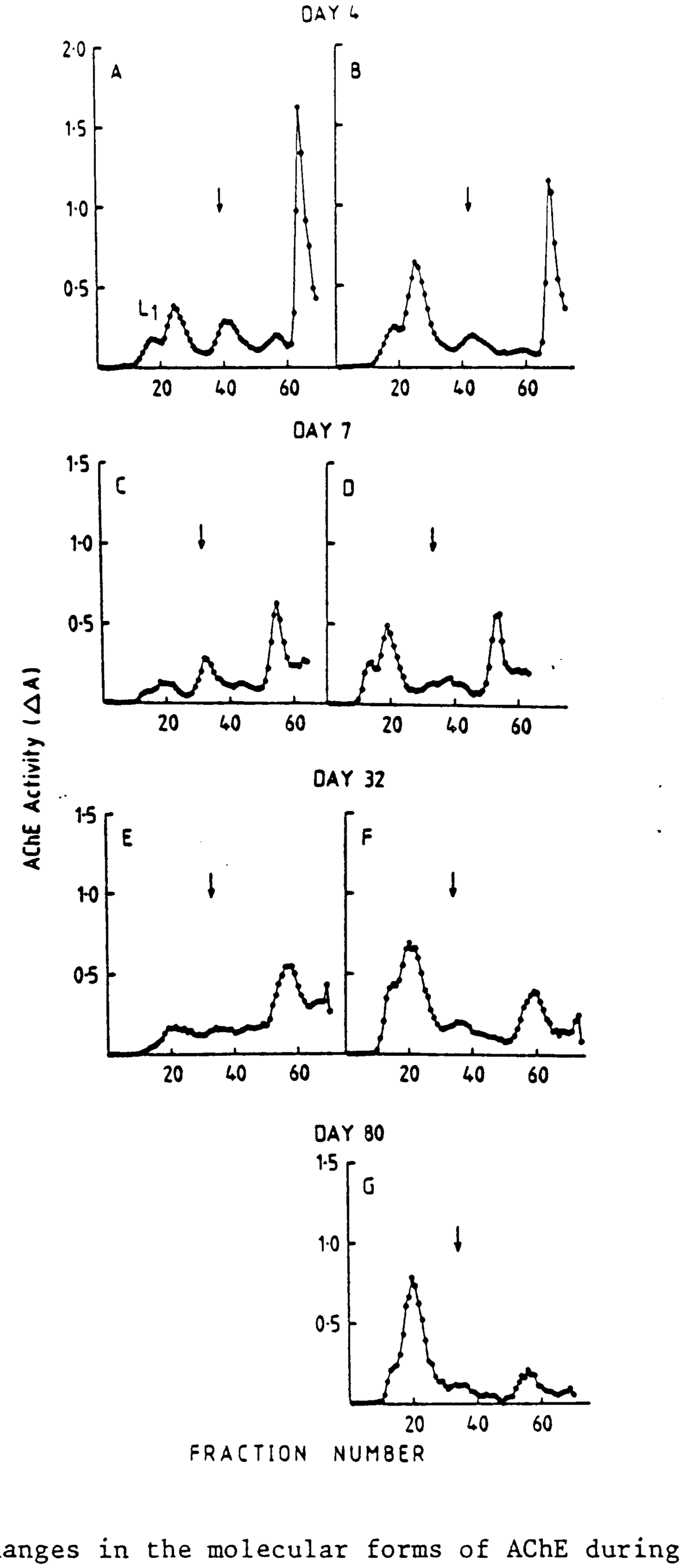


Figure 10. Changes in the molecular forms of AChE during maturation ex ovo of normal and dystrophic pectoral muscle.

The age of the muscle is shown above each pair of profiles of normal (A,C,E) and dystrophic (B,D,F,G) muscles. IF: A, 0.75; B, 0.75; C, 1.0; D, 1.0; E, 4.7; F, 2.0; G, 1.0.

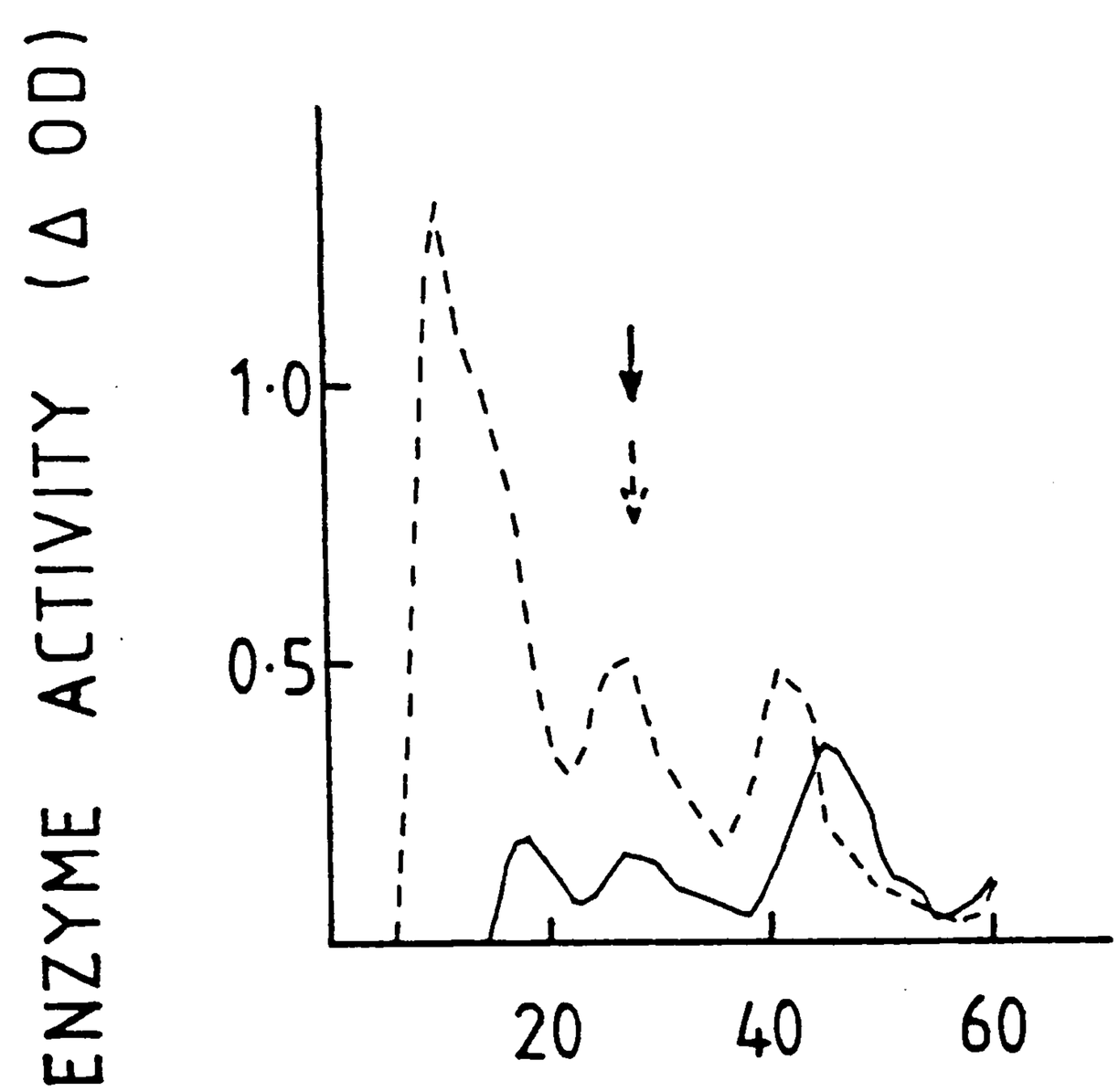


Figure 11. AChE and ψChE molecular forms in a dystrophic pectoral muscle on d600.

Solid line (———) represents AChE; dashed line (- - -) represents ψChE. IF: AChE, 16.5; ψChE, 62.9.

are not yet innervated, histochemical stain for AChE first appears around d5-6 and it is diffusely located in the cytoplasm. On d8 *in ovo* 80% of the AChE is found in this study to exist in the L_2 form, with very little H_2 form apparent. During further embryonic development, the proportion of H_2 AChE progressively increases.

As development proceeds, staining for AChE also increases in the region of the future endplate, while decreasing in the cytoplasm (Mumenthaler & Engel, 1961). The first discrete shapes indicative of localized endplate AChE were detected by Mumenthaler & Engel on d14 *in ovo* in the paravertebral muscle; and by Sisto Daneo & Filogamo (1975) on d13-14 in the PLD and ALD muscles. This correlates well with the increase shown here of H_2 AChE to 20% on d14 in the pectoral muscle. The proportion of H_2 AChE increases steadily as innervation is completed (which occurs later in the pectoral, *ca* d16, than in other muscles, Ashmore *et al*, 1978). When endplates are well established (d17-19), more enzyme activity exists in the H_2 form than in any other; AChE stain was reported to occur only at the endplate at this stage. The fact that only 15% of the AChE activity exists in the soluble L_2 form on d19 (Table 5) correlates well with reports showing that no AChE stain occurs in the cytoplasm. Thus, it appears that the proportion of H_2 AChE increases with the degree of muscle innervation during embryogenesis.

The important effect of the nerve in regulating the H_2 form of AChE in muscle has been examined in fully developed muscles ex ovo and in tissue culture. Denervation of several types of muscles in the chicken causes the total disappearance of the H_2 form but high total levels of AChE (Sketelj *et al*, 1978; Koenig & Vigny, 1978b; Silman *et al*, 1979). Denervation also causes a change in the location of AChE: from its being restricted at the endplate to its appearance extrajunctionally on the membrane. Direct electrical

stimulation of such denervated muscle reverses these effects, lowering the levels and reducing the amount of extrajunctional enzyme (Weidorff & Wilson, 1977). When rat soleus muscle is denervated after first being innervated at an ectopic site, AChE staining and the H form inititally disappear from the original endplate; subsequently, however, both reappear at the site of the original, as well as the ectopic, endplate (Weinberg & Hall, 1979). This is in contrast to the total disappearance of H AChE from completely denervated muscle. Therefore, the influence of the nerve (or contraction) in mature muscle appears to be to stimulate the production of the H_2 form of AChE and to restrict its localization at the endplate.

Experiments using rat or chick myotubes grown in tissue culture show, conversely, that the presence of nerves stimulates the production *in vitro* of the heaviest form of AChE (Koenig & Vigny, 1978a; Koenig, 1979; Rotundo & Fambrough, 1979). The increase of H_2 AChE during myofibre differentiation thus appears related to the presence of nerves.

How nerves stimulate the production of the forms of AChE is not clear. A diffusible molecule (other than ACh) which affects AChE synthesis in muscle is believed to be released from nerves when they are stimulated (Fernandez & Inestrosa, 1976; Younkin *et al*, 1978; Rathbone *et al*, 1979 a & b). Rathbone *et al* have identified a small protein (20,000 MW) from sympathetic dorsal root ganglia or chick brain which, when added to newt muscle in tissue culture, maintains the production of the H and M AChE forms. The same effect is produced when dorsal root ganglia are co-cultured with muscle, even when separated by a Millipore filter.

Several proteins involved in neurotransmission (AChE; ACh receptors; acetyl-cholinetransferase, AcCh) increase similarly during muscle development and it has been suggested that they are regulated by a similar mechanism. Simantov & Sachs (1972), however, showed that the

control of AChE and AcCh are not identical. They isolated a clone of neuroblastoma cells which produces a very low level of AChE while producing normal levels of AcCh. They proposed that the production of AChE (a membrane-bound enzyme) may be associated with the structure of the surface membrane, since the low AChE containing clonal line also had a low affinity for the surface of the tissue culture dish.

A similar reason (i.e. a change in the structure of the surface membrane) has been suggested for the accumulation of ACh receptors at the endplate area of myotubes grown with nerves in tissue culture (Anderson & Cohen, 1977). They proposed that the point of contact of a nerve (due to a ligand interaction) with an area of the muscle cell surface membrane produces a focal point for the attachment of micro-filaments from the interior of the muscle cell. This, in turn, is thought to control the mobility of proteins (e.g. receptors) in the membrane. Thus, the point of nerve contact causes an accumulation of receptors which develops into an endplate.

A structural feature of the membrane which reputedly is important in the appearance of the H_2 form of AChE at the endplate is the basal lamina. This structure is presumed by McMahan et al (1978) to provide a collagen milieu for the attachment of the H_2 form by its collagen tail. It is unclear if such a basal lamina is necessary for the appearance of AChE at the synapse. A partial basal lamina develops at the endplate area where nerve terminals contact muscle cells when grown in culture (personal communication from Dr. Gerald Fishbach). A complete basal lamina covers the myofibre in vivo and this fuses with the basal laminae from the Schwann cell and axon at the endplate junction. The cell type which is the source of the basal lamina is not certain. Apparently, it is not the Schwann cell, since there were no Schwann cells in the cultures of chick nerve and muscle cells which develop a partial

basal lamina. The stimulus for the proliferation of basal laminae may be contact by the nerve terminal, thus explaining why it originates from the endplate area of differentiating myofibres *in vitro*.

If the basal lamina is necessary for positioning AChE at the endplate, it may provide a regulatory mechanism for controlling the timing of the appearance of the H form *in ovo* or *in vitro*. H forms might, for instance, be produced at endplates by the addition of tetramers to collagen tails which are present in the basal lamina. Hence, no H form could be produced until muscle cells develop a basal lamina. Alternatively, a stimulus from the nerve may cause the parallel synthesis of basal lamina and of H forms. Such a stimulation of the production of H forms may be due either to initiating the synthesis of the tail (also collagen) or to the assembly of tetramers and tails. According to this alternative, the H form could be produced on internal membranes and then inserted onto synaptic basal laminae later.

Since a small amount of H_2 AChE exists as early as d8 *in ovo*, it would be interesting to know if any basal lamina also exists at this stage. If not, the synthesis of the H_2 form would not be dependent on the presence of basal laminae (or due to the existence of preformed tails in that structure). Instead, the second idea proposed above would be more probable - that the production of H_2 AChE and basal lamina are independent but stimulated during the same embryonic stage as part of the coordinated development of the endplates.

The synaptic region of the basal lamina of mouse muscle cells was reported to differ from extrajunctional basal lamina insofar as it contains no collagen (Zachs *et al*, 1973). This surprising finding contradicts the rationale for the proposed mode of attachment of H AChE to the basal lamina. It is unclear whether this finding

is an artifact or whether it is also true in other species. Nevertheless, since synthesis of the basal lamina originates from the endplate area, this structure appears to be closely associated with synaptic development.

Finally, although both AChE and receptors appear at a similar stage *in ovo*, they are probably not regulated by the same mechanism. Evidence of this comes, first, from a clonal line of neuroblastoma cells which produces 80 fold less AChE than normal, while producing normal levels of receptors (Simantov & Sachs, 1973). Thus, regulation of the synthesis of these proteins can be separated. Second, the presence of receptors alone is not sufficient to enable AChE synthesis. Muscle contractility is also necessary in regulating normal AChE synthesis. When the normal development specifically of muscle contractility is prevented *in ovo*, AChE is not synthesized normally, although receptors do develop (Vrbová *et al*, 1978). Even in the absence of synapses, normal myotubes can contract spontaneously *in ovo* or *in vitro*. Direct electrical stimulation of myotubes in tissue culture regulates the level of AChE by reducing the amount of enzyme produced and released by the cells. A blockage of such spontaneous contractions of muscle cells *in vitro* causes a loss of the enzyme regulation which can be induced by electrical stimulation; this decontrol results in increased AChE levels (Wilson & Walker, 1975). The regulation of AChE during normal muscle cell differentiation, although correlated with nerve contact and synapse formation, may be more directly due to another reason: either a factor released from the nerve or the regulatory effect of contraction. These studies confirm that the appearance of H_2 AChE *in ovo* is correlated with the development of the endplate.

Whether this is dependant on the presence of basal laminae, however, requires further investigation.

Dystrophic pectoral muscle

The pectoral is the largest muscle in the chicken and is the major one involved in the motions needed for a chicken to right itself from a prone position (i.e. to flip). This muscle is affected by dystrophy, functionally (e.g. reduced flip numbers) and biochemically (see 1.4.2.3), within the first several weeks ex ovo. The genetic lesion has further been shown in this study to be operative by d4 ex ovo. Abnormal control of AChE causes increased synthesis, specifically, of the L_2 subunit, with later accumulations of excess heavier forms of the enzyme. This appears to be the earliest qualitative, as well as quantitative, biochemical manifestation of the dystrophic lesion.

It has been suggested that dystrophy is a result of a retarded maturation of the white, fast twitch muscle fibres. This does not appear to be the case regarding AChE, since the progression of AChE forms during embryonic development is normal: the usual reduction of L_2 and its conversion into H_2 occurs by d19 in ovo. The fact that AChE levels are lower than normal in ovo may, however, reflect some abnormality during the early development of dystrophic muscle. The dramatic decontrol of enzyme synthesis, though, only occurs after hatching.

This abnormal control of AChE forms in dystrophic muscle sheds some light on the normal synthesis of this enzyme. First, the increase in M and H forms only appears several days after the initial elevation of L_2. This implies that a lag period is necessary for the processing of the dimer into larger forms. This

conforms to the proposed model of AChE synthesis:

monomer → dimer → tetramer → tailed forms.

Second, the L_2 form always remains the major form in dystrophic muscle. Since all forms are not increased equally, this implies the existence of control points in the synthesis of the various forms. For instance:

1) An enzyme might be necessary to join dimer into tetramer. Rieger et al (1976) suggested such a control point for the regulation of AChE synthesis in mouse neuroblastoma cells. They found that DNA dependent RNA synthesis was necessary for the production of the M (10S) form, but not of the L (4S) form. They suggested that the conversion of the L form into tetramers requires the translation of an essential messenger RNA: either for a new polypeptide chain which is involved in the linkage of the subunits or for an enzyme that initiates subunit polymerization into the M form. If such a protein involved in dimer conversion were produced normally in dystrophic muscle, while the synthesis of L_2 is decontrolled, a net excess accumulation of L_2 would result, even though higher than normal levels of M and H might also eventually appear.

2) The total amount of the heavier (M and H) forms might be limited physically by the amount of available membranes on which these forms are accommodated. Small, more soluble forms (L_2) are synthesized in the interior of the cell. Membrane-bound forms (M and H) are probably transported to the cell surface via the internal membranes (as discussed in 1.1.4). The fact that staining for AChE increases around the endplate in dystrophic muscle fibres (Patterson & Wilson, 1976a&b) illustrates that there is more AChE located extrajunctionally on the surface; this may correspond to

the higher than normal amounts of the H_2 form reported here. Less than normal amounts of AChE, however, have been reported in microdissected endplates from dystrophic muscle (Jedrzejzcyk et al, 1973). Since AChE is spread over a larger area than usual around the endplate, much of the excess enzyme may actually be outside the endplate and may not have been removed in the microdissection experiments.

The excess levels of M AChE may be located on internal membranes, or perhaps on the surface membrane as well. Patterson & Wilson (1976b) found increased perinuclear AChE stain in areas away from the endplate of dystrophic fibres which might account for the excess M form. In neuroblastoma cells, M AChE has been located on the cell surface (Vigny et al, 1979b) so that this form may also be located in the muscle cell surface. If the internal membranes of dystrophic fibres are fully covered with AChE, this may prevent further conversion of L_2 into M, either through a feedback mechanism or because there is no available membrane on which to deposit more M form.

3) Finally, conversion of M into H AChE might be limited by control of the production of the tail or the assembly of the latter with tetramers. This, again, could be controlled by the production of an enzyme or polypeptide necessary for such an association like that suggested for the conversion of L_2 into M.

In very old dystrophic pectoral muscle (e.g. d600) the pattern of AChE forms reverts to normal, with H_2 predominating. Although the levels of AChE are also lowered, this is due to a reduction in the number of muscle fibres, not to decreased enzyme production, since AChE concentration per gram of muscle fibre is still elevated ca 45 fold. No reason is apparent which could explain the normalized distribution of AChE forms. Perhaps the actual

rate of enzyme overproduction is slowed enough to enable the conversion of L into M and H forms to keep pace with the overproduction of L_2. This would argue in favour of suggestions 1 or 3 above - that steps which occur after the synthesis of L_2 are rate limiting in the production of M and H forms.

4.2.2 ψChE

4.2.2.1 Results

Enzyme levels

ψChE levels change in normal pectoral muscle the same way that AChE levels change during development: they increase at the end of embryonic development and decrease sharply after hatching (Table 8, Fig. 7). They are maintained at constant, low levels during further maturation ex ovo (Table 7). The concentration of ψChE in normal muscle is much less than that of AChE (by ca 10 fold) at all ages. Like AChE, ψChE also increases in dystrophic muscle between d14 and d19 in ovo; ψChE levels are also lower than normal on d19. Unlike AChE, ψChE concentration increases steadily in dystrophic muscle just after the time of hatching and is maintained consistently elevated to d260. Although it declines to ca 10X normal by d600, ψChE concentration is actually ca 90X normal when calculated per gram muscle fibre, as described for AChE (see 4.2.1.1). Thus, the abnormal regulation of ψChE in dystrophic muscle is not identical to the alteration in the control of AChE. The increase in enzyme production is greater for ψChE during the initial stage ex ovo, causing a sudden elevation in its concentration immediately after hatching. ψChE levels also fluctuate less than AChE ex ovo and are consistently elevated up to d260. These differences result in changing ratios of AChE and ψChE in the dystrophic pectoral muscle, particularly in older muscle where relatively higher ψChE levels occur.

It was, therefore, considered whether or not ψChE is converted into AChE, as proposed by Koelle (see 1.2.4). A homogenate of dystrophic muscle, with significant levels of AChE and ψChE, was incubated at $4^{o}C$ and at room temperature with ATP ($10^{-3}M$) in case such a conversion of enzymes occurs by phosphorylation. Assays were done at 0 and 43 hours (Table 3). No appreciable change in the activities of either enzyme was detected, thus proving both that the enzyme activities are fairly stable and that no interconversion of the two enzymes is apparent *in vitro*. Fluoride ion was added to the ATP/enzyme mixture at 0 hours to promote phosphorylation. This attempt was aborted when fluoride was found to inhibit most of the AChE activity; a phenomenon also reported by Silver (1974) to occur with AChE, in general. No ψChE activity was inhibited by fluoride, which is another indication that the enzymes are two different proteins.

Very old dystrophic muscle is primarily composed of fatty tissue which increasingly replaces muscle throughout the dystrophic process. Lower than normal levels of ψChE have been reported to occur in the fat cells of genetically obese mice (Kutty *et al*, 1979); fat cells also show stain for ψChE in denervated rat muscle (Eränko & Teräväinen, 1977). If fat cells in the dystrophic chicken produce ψChE, this might explain the variable proportions of AChE and ψChE during different stages of dystrophy. Body fat from the back of the dystrophic chicken (12 weeks *ex ovo*) was homogenized and assayed, but revealed no ψChE activity:

AChE, 0.185 U/g

ψChE, 0 U/g.

Although there is the possibility that fat cells in muscle do not behave identically, body fat does not produce ψChE in the dystrophic chicken.

Enzyme forms

The progression of the forms of ψChE during embryonic development of normal pectoral muscle is similar to that of AChE (Fig. 12; Tables 8 and 9): light forms predominate during early embryonic ages, while M and H gradually increase during later development. A similar pattern occurs in dystrophic muscle except that, as reflected in the lower overall enzyme concentration, the total amount of each form is less than normal in ovo. There are two main differences from the progression of AChE forms in ovo. First, M is the major form of ψChE throughout embryonic development. There is a considerable amount of L at d8. This appears to be converted later into heavier forms, as the amount of M remains stable and a progressive increase in H_2 occurs.

A second significant difference between the regulation of ψChE and AChE forms is that the proportion of activity in the H_2 form is always lower for ψChE, being a maximum of 40% at d19 in ovo. This proportion decreases ex ovo in normal and dystrophic muscle. H_2 is barely detectable in mature normal muscle, where M appears to be the major form (Fig. 12G).

ψChE levels, although quite low in normal pectoral muscle, are high in normal plasma. Very extended incubation times (48-96 hours) were needed to develop gradients of normal muscle in order to detect any peaks of ψChE activity. Therefore, normal muscle was perfused to remove any possible contamination due to ψChE in the blood. The actual pattern of ψChE forms produced in normal muscle is quite different from that seen in unperfused muscle (Fig. 13). L_1 is, in this case, the major form. Smaller and nearly equal amounts of M and H_2 are also present. A great proportion of the ψChE activity detected on gradients of unperfused normal muscle is, therefore, due to the blood plasma (where M is the major form, as discussed in 6.3.1).

TABLE 8

THE CHANGING FORMS OF ψChE IN

EMBRYONIC PECTORAL MUSCLE

Age (d)	n	Total enzyme activity (U/g)[a]	% of activity[b] in: L (L_1+L_2)	M	H_2
Normal					
8	(4)	0.23	41 (26+15)	52	4
14	(3)	0.26	26 (17+ 9)	53	16
16	(3)	0.24	11 (4+ 7)	57	27
19	(5)	0.44	17 (12+.5)	38	39
Dystrophic					
19	(5)	0.23	25 (17+ 8)	31	36

[a]Enzyme activity represents the mean of the number (n) of different birds tested ± the standard error of the mean (S.E.M.).

[b]Values represent the mean % of enzyme activity in each form for n birds tested.

TABLE 9

CHANGES IN THE LEVELS OF ψChE FORMS IN DEVELOPING NORMAL AND DYSTROPHIC CHICK PECTORAL MUSCLE

		Day 19 in ovo
Total ψChE activity (mU/muscle)	N	222
	D	111
L_1		
% of total ψChE activity	N	13
	D	17
Total (mU/muscle)	N	29
	D	19
% change from normal (total L_1)		-34
M		
% of total ψChE activity	N	30
	D	31
Total (mU/muscle)	N	67
	D	34
% change from normal (total M)		-49
H_2		
% of total ψChE activity	N	36
	D	36
Total (mU/muscle)	N	80
	D	40
% change from normal (total H_2)		-50

The terms L_1, M and H_2 are defined in Table 4. N = normal; D = dystrophic.

TABLE 9 -- Continued

Day 4	Day 7	Day 32
143	239	<300
208	643	18,160
16	9	6
42	38	39
23	22	<18
87	244	7,082
+282	+1,035	>+3,920
35	38	45
18	29	17
50	91	<135
37	186	3,087
-25	+105	>+2,190
31	22	12
20	17	22
44	53	<36
42	109	4,000
-5	+107	>+1,100

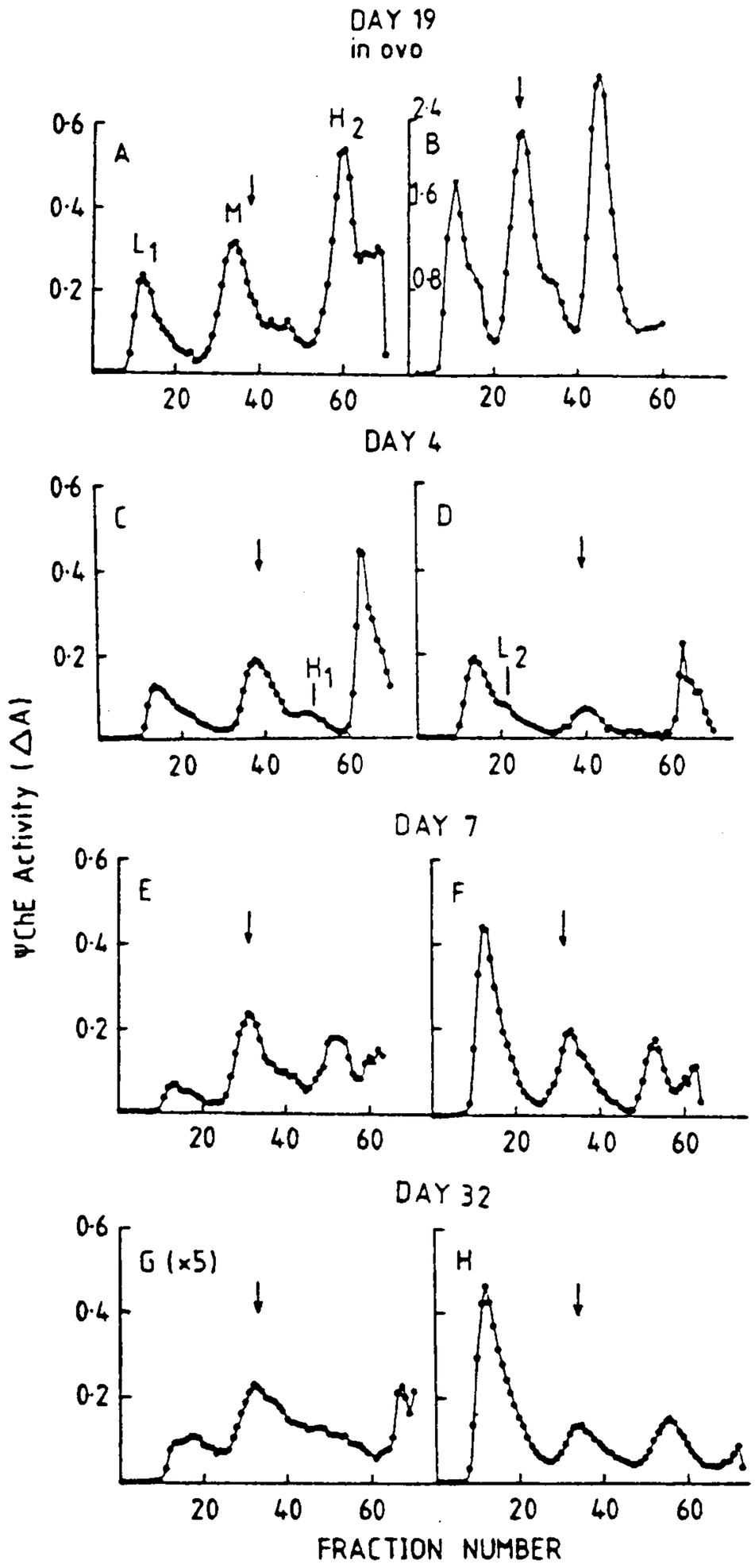


Figure 12. The changes in ΨChE molecular forms during development of the pectoral muscle in normal and dystrophic chickens.

The age of the normal (A,C,E,G) or dystrophic (B,D,F,H) muscles is shown above each pair of profiles.

Long incubations were employed in profiles B (hence the different y axis) and G (as indicated by "X5").

IF: A, 4.2; B, 51.3; C, 8.8; D, 14.9; E, 15.0; F, 6.0; G, 33.8; H, 6.0.

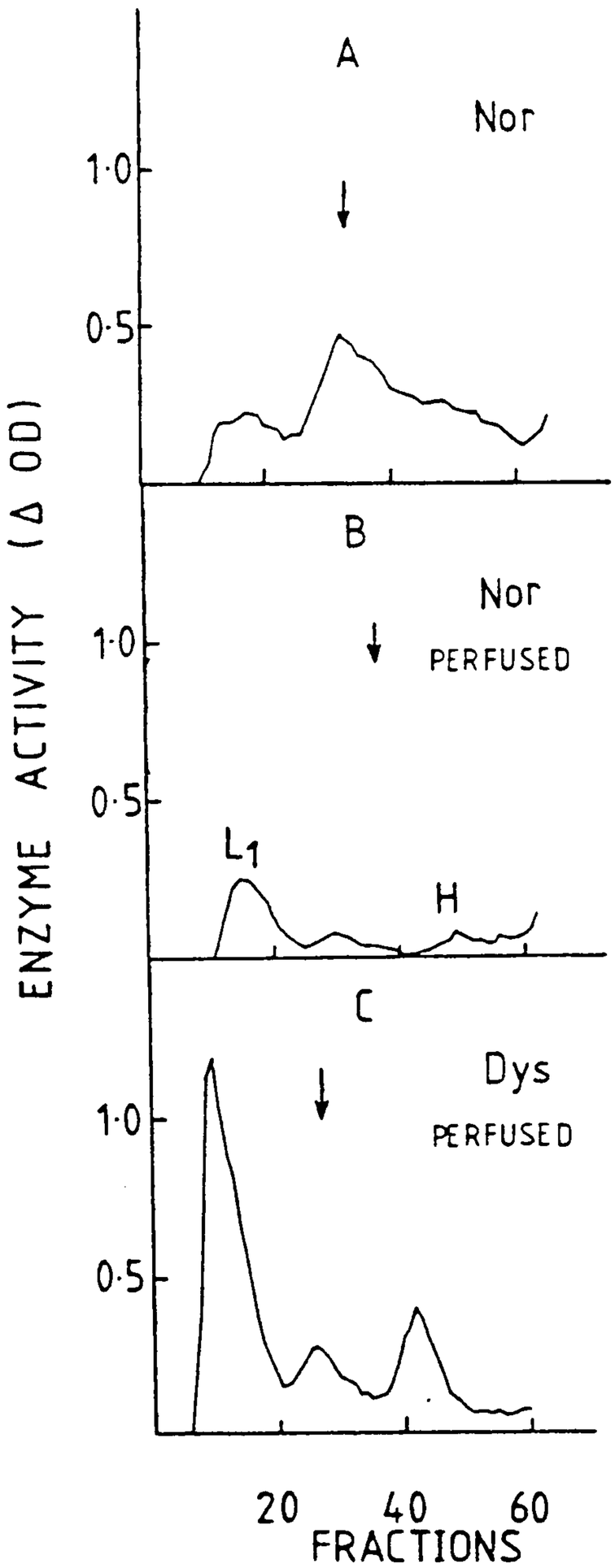


Figure 13. The effect of perfusion on the molecular forms of ψChE detected in mature normal and dystrophic pectoral muscles.

Nor = normal; Dys = dystrophic. A sucrose gradient profile of an unperfused, normal control pectoral muscle is shown in (A). Each profile represents muscle from only one bird.
IF: A, 33.8; B, 14.4; C, 5.5.

ψChE is clearly overproduced in dystrophic muscle immediately after hatching: excess amounts of L_1 are present on d4, while subsequently increased levels of M and H_2 occur. L_1 remains the predominant species throughout development. Prolonged gradient incubation periods, as needed to develop normal muscle gradients, were not necessary to detect ψChE activity on gradients of dystrophic muscle. Perfusion makes little difference to the ψChE profile (Fig.13) and, therefore, was not routinely employed with dystrophic muscles.

4.2.2.2 Discussion

ψChE has been studied most often as the more soluble enzyme (e.g. from plasma or pancreas). When detected in muscle by histochemical staining ψChE, like AChE, has been localized at the endplate. (Silver, 1963; Patterson & Wilson, 1976a) Its occurence as multiple forms, especially as a tailed form, has not been investigated fully in muscle. It is shown here that ψChE has an important collagenous component. Also like AChE, it may be associated at the muscle endplate by the attachment of its tail. Since the H_2 form is the least prominent one in both normal and dystrophic muscle, the question arises of whether H_2 is the only form present at the endplate. Incubation times needed to develop ψChE activity at endplates are often reported to be ca 8-10 times those needed to develop AChE activity. This is similar to the relative concentrations of total AChE and ψChE activity in normal muscle. Considering that H_2 is the major form of AChE, but the minor ψChE form, there is roughly 30 fold more H_2 AChE than ψChE. Therefore, it seems likely that lighter ψChE forms, in addition to H_2, may exist at the endplate.

ψChE is more prominent around the endplate in dystrophic muscle than in normal muscle (Patterson & Wilson, 1976a&b). It could be overproduced inside the cell and deposited at the endplate by

a process similar to that discussed for AChE. The importance of neural control in the synthesis of ψChE in muscle cells is demonstrated by the effect of denervation: total enzyme levels increase, L_1 is particularly overproduced, but the H_2 form disappears totally (Patterson & Wilson, 1976b; Silman et al, 1979). Therefore, ψChE resembles AChE in its location at muscle endplates and in being regulated by the nerve.

It is not clear why the control of the major enzyme form in normal, mature pectoral muscle is different for AChE and ψChE. The proportions of the various ψChE forms change in ovo like those of AChE forms. Mostly lighter forms are synthesized at early stages, but H_2 increases by d19. However, H_2 ψChE is never as predominant as it is for AChE. The order of the appearance of the ψChE forms in ovo indicates that it may be synthesized like AChE: L → M → H. This is most noticeable during the period from d8 to d16: while the total ψChE concentration and the percentage of M remain constant, H_2 increases in proportion to the decrease in L_1.

Control points, like those suggested in AChE synthesis, for the conversion of monomer into tetramer and tetramer into H_2 may also apply in the synthesis of ψChE. A notable difference between ψChE and AChE is found in the major light subunit: L_1 for ψChE and L_2 for AChE. This may reflect a difference in the relative stabilities of the two monomers and dimers. The inter-subunit bonds of the ψChE dimer may be more labile than those of L_2 AChE such that ψChE L_2 dissociates more easily into L_1 monomers. Alternately, a different control point in the synthesis of the ψChE tetramer may restrict the conversion of L_1 into dimer; whereas in AChE synthesis, the control may occur at the point of the conversion of L_2 to M.

In dystrophic muscle the excess production of ψChE, like AChE, is also mainly of the L forms, although there is much more L_1

than in the case of AChE. This occurs by d4 ex ovo, while increased M and H forms only appear later (Table 9). That a lag period is necessary between the increased production of L and heavier forms is further evidence that the synthesis of ψChE involves a sequential association of smaller subunits into larger forms. The L form of ψChE may be the major form in both normal and dystrophic muscle because the conversion to M and H forms is slower than the synthesis of L forms. The functional significance of the accumulation of the light, presumably cytoplasmic, form of ψChE, as opposed to the H_2 membrane-bound form of AChE is unclear.

The unusually high levels of ψChE in relation to AChE which occur in dystrophic muscle between d60 and d270 do not appear to be due to ψChE production by fat cells as judged by enzyme assays of body fat. However, ψChE was shown to appear abnormally in Schwann cells and fat cells of rat muscle upon denervation (Eränko & Teräväinen, 1977); a similar phenomenon cannot, therefore, be discounted in such cells of dystrophic muscles which are also unhealthy. It could also be due to the production of ψChE by connective tissue which, along with fat, replaces muscle fibres as dystrophy progresses. Fibroblasts are the probable source of the ψChE activity in muscle cultures in vitro: ψChE increases with age in cultures of muscle cells as the proportion of fibroblasts also increases (Weinstock et al, 1978).

Another explanation for the variable ratios of excess AChE and ψChE is that the dystrophic lesion may not affect both enzymes identically at different times during the course of dystrophy. The forms of ψChE, for example, appear to be regulated abnormally at d600, while AChE forms are regulated normally. Alternatively, ψChE may not be released from the dystrophic muscle cell, although AChE is released. While ψChE is generally considered to be a relatively soluble enzyme, little is known of its internal location and it could be sequestered inside the cell, unavailable for release. It is felt, however, that another, more likely

explanation is that a non-muscle cell type, such as connective tissue or fat, which increases in dystrophic muscle with age, produces ψChE which is not released.

Thus ψChE from muscle has a structure, as well as a function, like AChE. Since the synthesis of its forms is similarly controlled in ovo and in dystrophic muscle, ψChE is often regulated in parallel with AChE. The two enzymes are not interconverted in vitro, thus discounting the idea that one is a precursor of the other. Normal muscle clearly produces ψChE by itself (e.g. in ovo and in dystrophic muscle) and synthesizes forms which are not present in the blood (e.g. H_2; see Chapter 6 for plasma forms). Such evidence also argues against Koelle's suggestion that the source of ψChE in muscle is absorption from the plasma (Koelle et al, 1979).

4.3 Posterior Latissimus Dorsi Muscle

4.3.1 Results

4.3.1.1 AChE

The levels of AChE change during development in the PLD muscle as they do in the pectoral muscle (Tables 10 and 11). AChE decreases immediately after hatching in the normal muscle to reach very low levels by d30. This decrease is slower in the dystrophic muscle and a later peak in AChE concentration occurs between d60 and d90, after which levels decrease.

The forms of AChE change the same way in the PLD as in the pectoral muscle: H_2 is the major form on d19 in ovo and throughout development of the normal muscle, whereas L_2 is the major form overproduced in the dystrophic muscle (Fig. 14). AChE forms are also regulated normally on d600 (Fig.15) in the dystrophic PLD, as in the dystrophic pectoral muscle.

TABLE 10

CHANGES IN THE LEVELS OF L_2 AND H_2 AChE IN DEVELOPING NORMAL AND DYSTROPHIC CHICK PLD MUSCLE

		Day 19 in ovo	Day 4
L_2			
% of total AChE activity	N	15	21
	D	16	27
Total L_2 (mU/muscle)	N	11.7	27.5
	D	11.6	34.5
% change from normal (total L_2)		-1	+26
H_2			
% of total AChE activity	N	45	42
	D	52	40
Total H_2 (mU/muscle)	N	35.0	59.9
	D	37.9	51.1
% change from normal (total H_2)		+8	-7
AChE concentration (U/g)[a]	N	8.65	7.07
	D	6.81	6.42

The terms L_2 and H_2 are defined in Table 4. N= normal; D = dystrophic.

[a]Activity is expressed as Units per gram wet weight of muscle.

TABLE 10 -- Continued

Day 7	Day 32	Day 60
16	14	10
26	21	31
13.9	24.0	45
25.5	97.0	605
+84	+304	+1240
48	55	60
44	44	33
41.8	94	270
43.2	203	644
+3	+116	+139
2.55	0.60	0.43
2.91	1.54	2.44

TABLE 11

LEVELS OF AChE AND ψChE IN DEVELOPING

NORMAL AND DYSTROPHIC PLD MUSCLE

Age (d)		AChE (U/g)[a] Normal	AChE (U/g)[a] Dystrophic	ψChE (U/g)[a] Normal	ψChE (U/g)[a] Dystrophic
7[b]		2.55	2.91	0.231	0.231
	n	4	4	4	4
32		0.60	1.54	0.041	0.318
	S.E.M.	±0.13	±0.34	±0.018	±0.027
	n	4	4	4	4
60		0.43	2.44	0.024	0.296
	S.E.M.	±0.02	±0.33	±0.011	±0.052
	n	3	3	3	3
90		---	2.66	---	0.483
	S.E.M.		±0.55		±0.105
	n		4		4
115		0.393	1.91	0.092	1.02
	S.E.M.		±0.24		±0.09
	n	1	5	1	5
600		0.133	0.598	0.013	0.263
	S.E.M.	±0.087	±0.013	±0.011	±0.142
	n	2	2	2	2

[a] Values represent the mean (±S.E.) of the total enzyme activity per gram wet weight of muscle for n birds tested.

[b] Values represent the automatic mean of the activity in muscles from n birds since muscles at this age were pooled before extraction due to their small size.

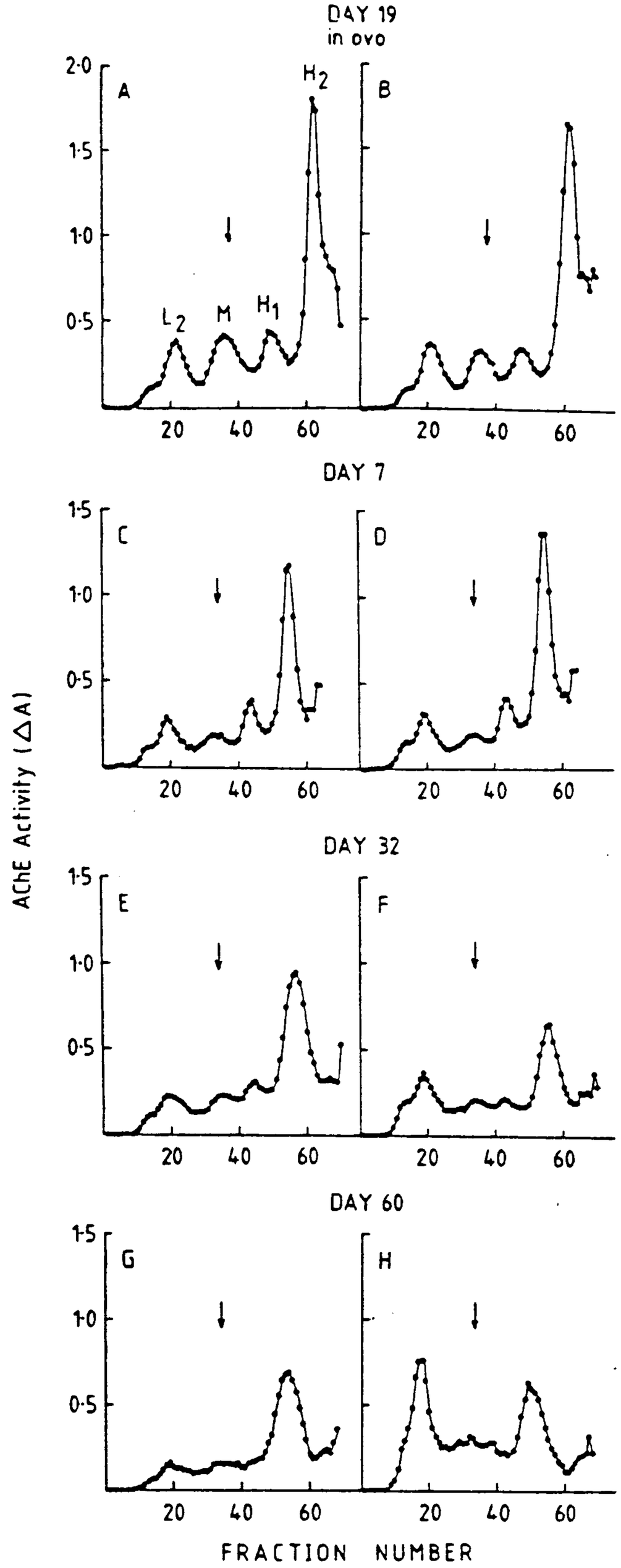


Figure 14. The changes in AChE molecular forms during development of the PLD muscle in normal and dystrophic chickens.

The age of the normal (A,C,E,G) or dystrophic (B,D,F,H) muscles is shown above each pair of sucrose gradient profiles.

IF: A, 0.53; B, 0.53; C, 1.13; D, 1.13; E, 2.69; F, 3.0; G, -; H, 1.5.

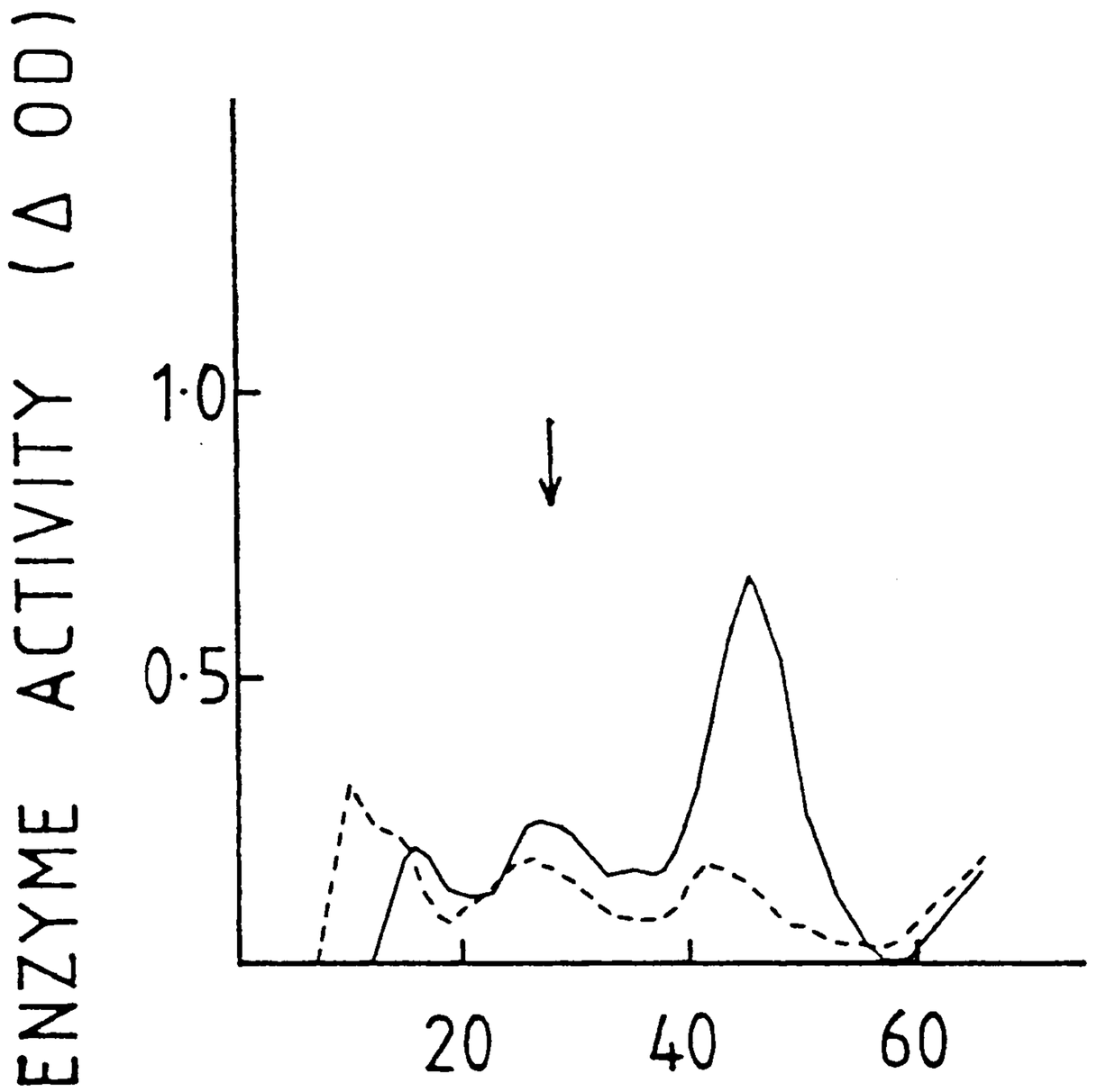


Figure 15. AChE and ψChE molecular forms in a dystrophic PLD muscle at d600.

Solid line (———) represents AChE; dashed line (- - -) represents ψChE.

IF: AChE, 19.5; ψChE, 143.0.

The main differences between the manifestation of dystrophy, as regards AChE and ψChE, in these two fast twitch muscles concern the magnitude and timing of the alterations. A direct comparison of the total amount of AChE per dystrophic muscle (Fig. 8) shows that the great increase in AChE production does not begin until after d7 in the PLD, compared to d4 in the pectoral; by d30 the increase is ca 4 fold greater in the pectoral, than in the PLD muscle. The disproportionate increase of L_2 is not as dramatic and occurs later in the PLD muscle. For example, L_2 only reaches a level of 31% by d60 in the PLD muscle, compared to a value of 51% on d32 in the pectoral muscle.

4.3.1.2 ψChE

ψChE activity decreases during muscle maturation ex ovo in the normal PLD muscle (Table 11) and is only a fraction of the level of AChE activity, as it is in the pectoral muscle. ψChE is similarly elevated ex ovo in the dystrophic PLD, as in the pectoral muscle, rising to levels more than 150% those of AChE around d120 but decreasing in the very old muscle.

The forms of ψChE are also regulated alike in pectoral and PLD muscles. At d18 in ovo enzyme activity is distributed fairly evenly between L_1, M and H_2, although there is slightly less H_2 (ca 20%).

Normal, unperfused, mature PLD muscle, like the pectoral, shows little ψChE activity on gradients except in the M region, but when perfused, ψChE is mostly in the L form (Table 12). The dystrophic muscle greatly overproduces all forms, especially L, even at d600 (Fig.15).

4.3.2 Discussion

The PLD is a fast twitch muscle of the chicken which attaches the wing to the thorax and is involved in raising the wing. It originates on the ligament connecting the first four thoracic vertebrae and

TABLE 12

THE CHANGING FORMS OF ψChE IN THE

DEVELOPING PLD MUSCLE

Age (d)	n	% of activity[a] in: L (L_1+L_2)	M	H	
18 in ovo					
Normal	4	36 (18+18)	26	20	
35					
Dystrophic	4	64 (32+32)	18	21	
80					
Normal (unperfused)	1	8	55	16	
Normal (perfused)	1	52	29	7 14	(peak)[b] (entire H region)
Dystrophic	8	50	29	21[c]	
600					
Dystrophic	2	76 (37+39)	14	4	
Normal	2	23 (11+12)	48	18	

Muscles were generally not perfused, except in the case of the normal muscle on d80.

[a]Values represent the mean % of enzyme activity in each form for n birds tested.

[b]Only a small and indistinct peak was apparent in the 15S area of the H region after 50 hours of incubation.

[c]A substantial and distinct peak (ca 15S) was easily distinguished after 5 hours of incubation.

inserts on the ventral surface of the humerus. Like the pectoral muscle, it is composed almost purely of white, fast twitch fibres.

The regulation of AChE and ψChE is similar in the PLD and pectoral muscles. The H_2 form of AChE is the major one in both of these normal muscles after hatching, while L_2 is overproduced in the dystrophic muscles. With regard to ψChE, L_1 is overproduced in the dystrophic muscle while barely being detectable in the normal PLD. The difference between the pectoral and PLD muscles is that dystrophy affects the PLD more slowly and less severely. Elevations in the enzymes are never as high in the PLD and they occur more gradually over the first several months. This confirms the results of other studies which have shown that the pectoral muscle exhibits a greater degree of hypertrophy and larger elevations of several biochemical markers than other muscles which are also affected by dystrophy.

Levels of both ψChE and AChE are decreased by d600 in the dystrophic PLD, as in the dystrophic pectoral muscle. AChE activity is found mostly in the H_2 form at this age, whereas the ψChE pattern continues to show excess levels of L_1. The dystrophic PLD is composed of ca 50% muscle fibre at d600 (data to be published in a thesis by Mr. J. Pizzey from this laboratory, Biochemistry Department, Imperial College) while the dystrophic pectoral muscle is only ca 10% muscle fibre. The elevation per gram muscle fibre of AChE in the dystrophic PLD at d600 is thus ca 8X normal, as compared to 45X normal in the dystrophic pectoral muscle at this age. Therefore, the PLD never becomes as severely afflicted by dystrophy as the pectoral muscle. Even so, regulation of the forms of the two cholinesterases is the same in both muscles at this late age: ψChE continues to be regulated abnormally, while AChE forms show a normal distribution, being predominantly H_2.

4.4 Flexor Carpi Ulnaris Muscle

4.4.1 Results

4.4.1.1 Levels of AChE and ψChE

The levels of AChE and ψChE were studied in detail during the embryonic development of the flexor carpi ulnaris (FCU) muscle (Fig.16). The wide range of AChE values found in this study early in ovo may be due to the greater individual variation which was reported by Goodwin & Sizer (1965) to occur during the early stages of embryonic development. It may also be due to the fact that these muscles were very difficult to handle. Being only several milligrams each, they were extremely jelly-like and hard to dissect accurately until well-developed at later ages (e.g. d17). Therefore, variations due to loss of tissue, incomplete dissection or evaporation would be more significant in these tiny muscles.

There is a definite increase in AChE levels in the normal muscle between d17 and d19 when the muscles become well formed. The levels in dystrophic FCU muscles do not increase similarly. The difference between these values is highly significant at d19 ($p<0.01$). ψChE levels, although lower than AChE, increase more evenly during embryonic development. In dystrophic FCU muscle, ψChE levels tend to be lower than normal, although this is not as significant on d19 as it is for AChE. In mature dystrophic muscle, the level of AChE is virtually normal on d37 but the ψChE level is ca 5 fold greater than normal.

4.4.1.2 AChE forms

The development of AChE forms in ovo is similar to the changes of AChE in the pectoral muscle: the L_2 form predominates at d16, while H_2 increases on d19 (Table 13). In developed muscle on d37

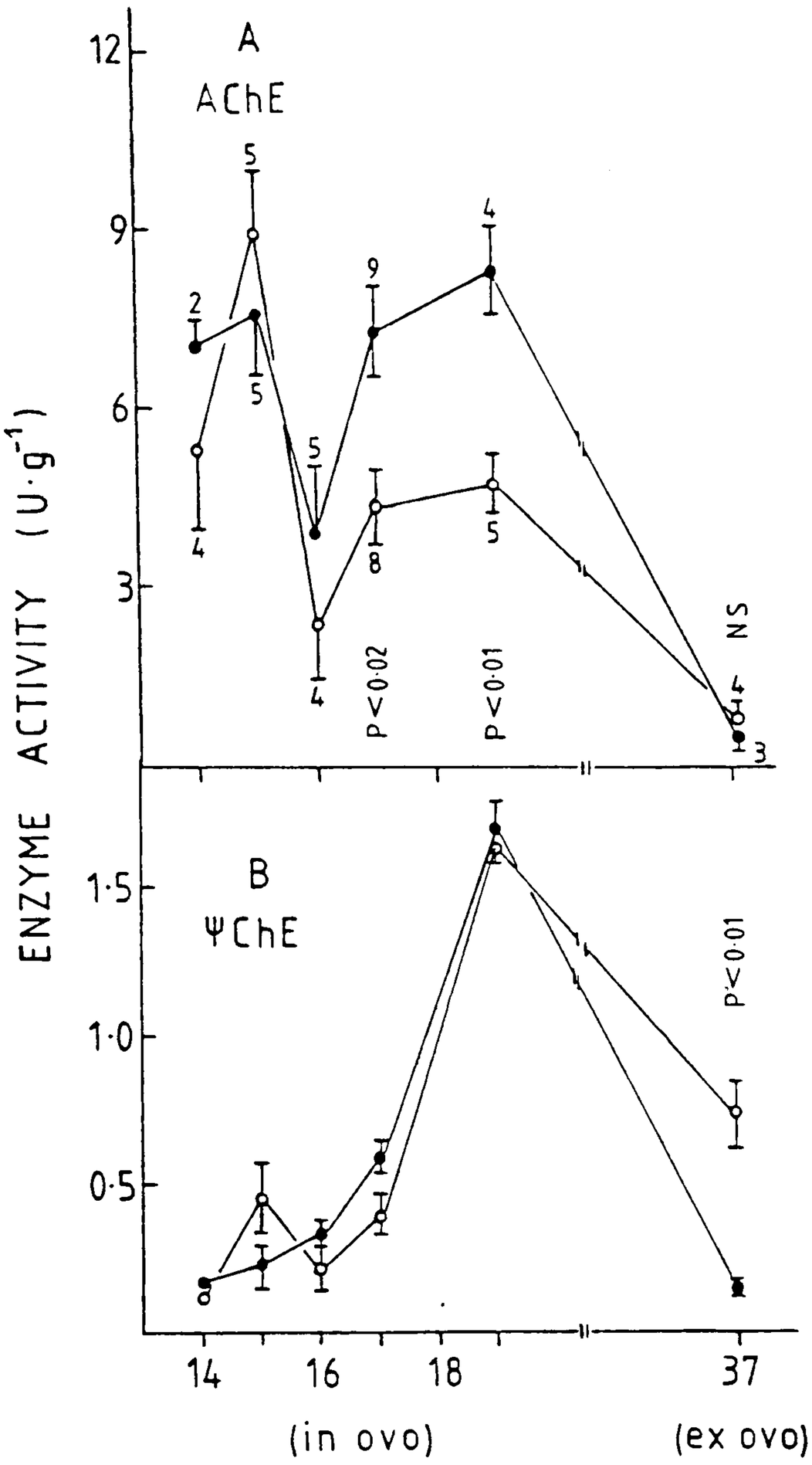


Figure 16. Changes in the concentrations of AChE and ΨChE in the developing FCU muscle in normal and dystrophic chickens.

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 is indicated, where appropriate, by P as determined by the student T test. Normal (●—●); Dystrophic (○—○).

TABLE 13

THE CHANGING LEVELS OF AChE AND ψChE FORMS IN THE FCU MUSCLE DURING DEVELOPMENT

A. AChE

Age (d)	% of activity in: L_2	M	H_2	
16 in ovo				
Normal	40	32	21	
Dystrophic	40	31	25	
19 in ovo				
Normal	39	28	30	
Dystrophic	41	28	28	
37 ex ovo				(H_1)
Normal	7	7	77	(9)
Dystrophic	48	15	31	(7)

TABLE 13 -- Continued

B. ψChE

Age (d)	L	(L_1+L_2)	M	H_2
16 *in ovo*				
Normal	39	(21+18)	46	10
Dystrophic	29	(15+14)	49	17
19 *in ovo*				
Normal	42	(36+ 6)	49	9
Dystrophic	46	(29+17)	43	11
37 *ex ovo*				
Normal	27		32	31
Dystrophic	74	(52+20)	14	11

ex ovo, the H_2 form is the major one (77%) in normal muscle. Although AChE levels are similar in both normal and dystrophic FCU, there is an increased proportion of L_2 (to 48%) in the dystrophic muscle.

4.4.1.3 ψChE forms

L and M are the major forms of ψChE to appear in embryonic FCU muscle at d16 and d19. H_2 is only a small proportion (ca 10%) of the total activity at either age. No differences were detected between the forms in normal or dystrophic muscle. The pattern of ψChE is quite different in developed, normal FCU muscle from that seen in the other two fast twitch muscles studied: all three major forms are present in equal amounts. However, in the dystrophic FCU, as in dystrophic pectoral and PLD, the L forms are greatly elevated (Table 13B).

4.4.2 Discussion

The FCU muscle is located in the lower part of the wing; it originates on the humerus and inserts on the carpometacarpus. It is a fast twitch muscle which is involved in folding the wing. Close examination of the levels of AChE and ψChE confirm the trend seen in the pectoral and PLD muscles that the dystrophic muscle does not regulate the levels of these enzymes normally in ovo. There is significantly less AChE present on d19 in the dystrophic muscle and there is generally less ψChE present throughout embryonic development. The FCU muscle however, is only mildly affected by dystrophy later in development. The similar, abnormally low synthesis of AChE and ψChE shown to occur in several embryonic fast twitch muscles may, thus, be an early manifestation of the dystrophic gene which affects all fast twitch muscles. It is not clear whether this is due to a specific effect on the control of AChE and ψChE or to an indirect effect on some other aspects of cell metabolism.

The normal FCU muscle does not regulate AChE and ψChE exactly the same as the other two fast twitch muscles studied. There is a slower development of H_2 as the major form of AChE; this does not occur *in ovo*, but sometime *ex ovo* before d37. This may be related to the timing of the functional development of this muscle. The FCU may not be among the earliest muscles to develop *in ovo* since it is a distal muscle which may not be as necessary as others (e.g. the pectoral) for the movements needed *in ovo* for hatching or for survival early *ex ovo*. Like other fast twitch muscles, however, H_2 does become the major form in mature normal muscle. Also like other dystrophic fast twitch muscles, the dystrophic FCU overproduces the L_2 form of AChE. The effect of abnormal enzyme control is not nearly as severe in the FCU muscle since levels are not even elevated by d37. This results in there being 50% less H_2 AChE than normal in the dystrophic FCU, as opposed to an excess of H_2 as found in other dystrophic fast twitch muscles.

Although beyond the scope of this study, it is intriguing to ponder what functional effects such a lower than normal level of H_2 AChE might cause in "mildly" dystrophic muscles. When AChE inhibitors are used to experimentally reduce enzyme activity at muscle synapses, a significant prolongation of the recovery time following electrical stimulation occurs (Magazanik *et al*, 1979). It is, therefore, possible that a reduced concentration of the endplate form of AChE at synapses of dystrophic muscle could also cause less efficient or slower muscle function.

Abnormal control of ψChE synthesis in the dystrophic FCU follows the pattern exhibited by other fast twitch muscles with the L forms being overproduced. The normal FCU muscle, however, does not regulate ψChE in the same way as other normal fast twitch muscles.

During embryonic development there are equally high levels of L and M forms, but only ca 10% is H_2. The latter form does not increase in ovo as it does in the pectoral and PLD. The embryonic pattern on d19 resembles that seen in the pectoral muscle on d8 or d14 in ovo, which may once again indicate that the FCU is slower to mature. Nearly equal amounts of all three forms of ψChE are found in the developed, normal muscle. Therefore, relatively more H_2, and also M, are produced in this muscle ex ovo than in other fast twitch muscles. The reason for this is not understood. It could be related to the fact that the PLD and pectoral muscles are almost purely composed of fast twitch fibres. All other so-called "fast twitch" muscles are, in fact, "mixed" with varying proportions of slow twitch or red, fast twitch fibres. The ψChE patterns in either of these fibres have not been determined and could be quite different from the pattern in fast twitch muscle. Thus, the FCU has a ψChE pattern which is unlike the two relatively pure fast twitch muscles previously reported here.

Abnormal enzyme regulation in the dystrophic FCU is not the same for AChE and ψChE since total levels of AChE are normal on d37 while ψChE is elevated 5 fold. A similar difference was noted in the very young dystrophic pectoral muscle: ψChE concentrations increase from hatching onwards, whereas AChE levels decrease initially (4.2.2.1). This may indicate that the dystrophic gene affects ψChE synthesis earlier than AChE.

4.5 Summary

The major forms of AChE present in fast twitch muscles are L_2, M and H_2 (sedimenting at 7S, 11S and 20S, respectively). Regulation of AChE in the mature, fast twitch muscles studied causes virtually all of the enzyme to be converted into the H_2, or so-called "endplate-specific", form. Synthesis of the H_2 form in ovo is correlated with the establishment of synapses. L_2 is the major form

produced early in embryonic development; increases of M and H_2 occur later. This suggests that AChE synthesis involves a progressive aggregation of the basic, light subunit into larger (M and H_2) forms. Such an order of synthesis of AChE forms is further supported by the case of dystrophic muscle which first overproduces the L_2 form, while excess M and H_2 forms only appear later.

The dystrophic gene has been shown here to be functional at least by d4 ex ovo, as evidenced by increased levels of L_2 in the pectoral muscle. It may even exert an earlier effect since AChE levels were found to be generally lower than normal in several different embryonic fast twitch muscles. The fact that L_2 is the main form of AChE overproduced by dystrophic muscle may indicate that a control point exists in the normal synthesis of larger AChE forms -- namely, the step involving aggregation of two dimers into the tetramer.

It has been shown that ψChE is definitely produced by muscle. Like AChE, this enzyme exists in several molecular forms: L_1, L_2, M, H_1 and H_2, although each form is slightly smaller (sedimenting at 4S, 6S, 10.5S, 14S and 19S, respectively) than the corresponding AChE forms. The heavy forms are sensitive to collagenase treatment, indicating that ψChE, like AChE, also exists as collagen-tailed forms.

The smaller (L and M) forms of ψChE are predominant early in ovo, but H_2 increases, as for AChE, near the time of hatching. Unlike AChE, however, ψChE synthesis is very reduced in mature, normal fast twitch muscles. Regulation of its forms is not the same as for AChE in the two, nearly pure fast twitch muscles: L_1 is the major form; smaller amounts of M and H_2 are also produced. Another distinctive feature of ψChE is that L_1 is the more prevalent light form, whereas L_2 is the predominant light form of AChE. However, like AChE,

the light form of ψChE is greatly overproduced, along with excess M, H_1 and H_2, in dystrophic fast twitch muscles. Thus, despite differences in the sizes of their forms, and in the regulation of AChE and ψChE in normal muscle, their similar deregulation in dystrophic muscle indicates that AChE and ψChE are closely related muscle enzymes.

CHAPTER 5

SLOW TONIC ALD MUSCLE

5.1 Results

5.1.1 AChE

The levels of AChE in normal and dystrophic embryonic ALD muscles are high just prior to hatching (d18) and they decrease after hatching as in fast twitch muscles, albeit more slowly (Fig.17). AChE levels are higher from d7 *ex ovo* and throughout further development in the ALD (Table 14) than in fast twitch muscles. AChE concentration only decreases *ex ovo* due to a dilution of enzyme in a rapidly increasing muscle mass. The total amount of AChE actually increases (*ca* 6 fold) in the normal ALD muscle between d7 and d30 (Fig.17B). Total AChE is constant in the ALD from 1 to 4 months. There are no significant differences between AChE concentration in ALD muscles from normal and dystrophic chickens at any age.

The same forms of AChE which occur in fast twitch muscles are also found in the ALD, except that their relative proportions are quite different (Fig.18). This difference is apparent as early as d18 *in ovo* when there is less H_2 than L_2 in the ALD; there is relatively more H_2 in the PLD which is the most comparable fast twitch muscle to the ALD because of its similar pattern of development and position. The proportion of H_2 AChE in the ALD decreases continually at all ages *ex ovo* until it is virtually undetectable after d113. L_2 is the predominant form at all ages; some M AChE is also present. The same changes in the forms of AChE occur in the ALD of dystrophic chickens (Fig.19).

5.1.2 ψChE

ψChE concentrations also decrease in the ALD muscle during the first week *ex ovo* (Fig. 17A). They remain at constant, low levels throughout further development and are similar at all ages in

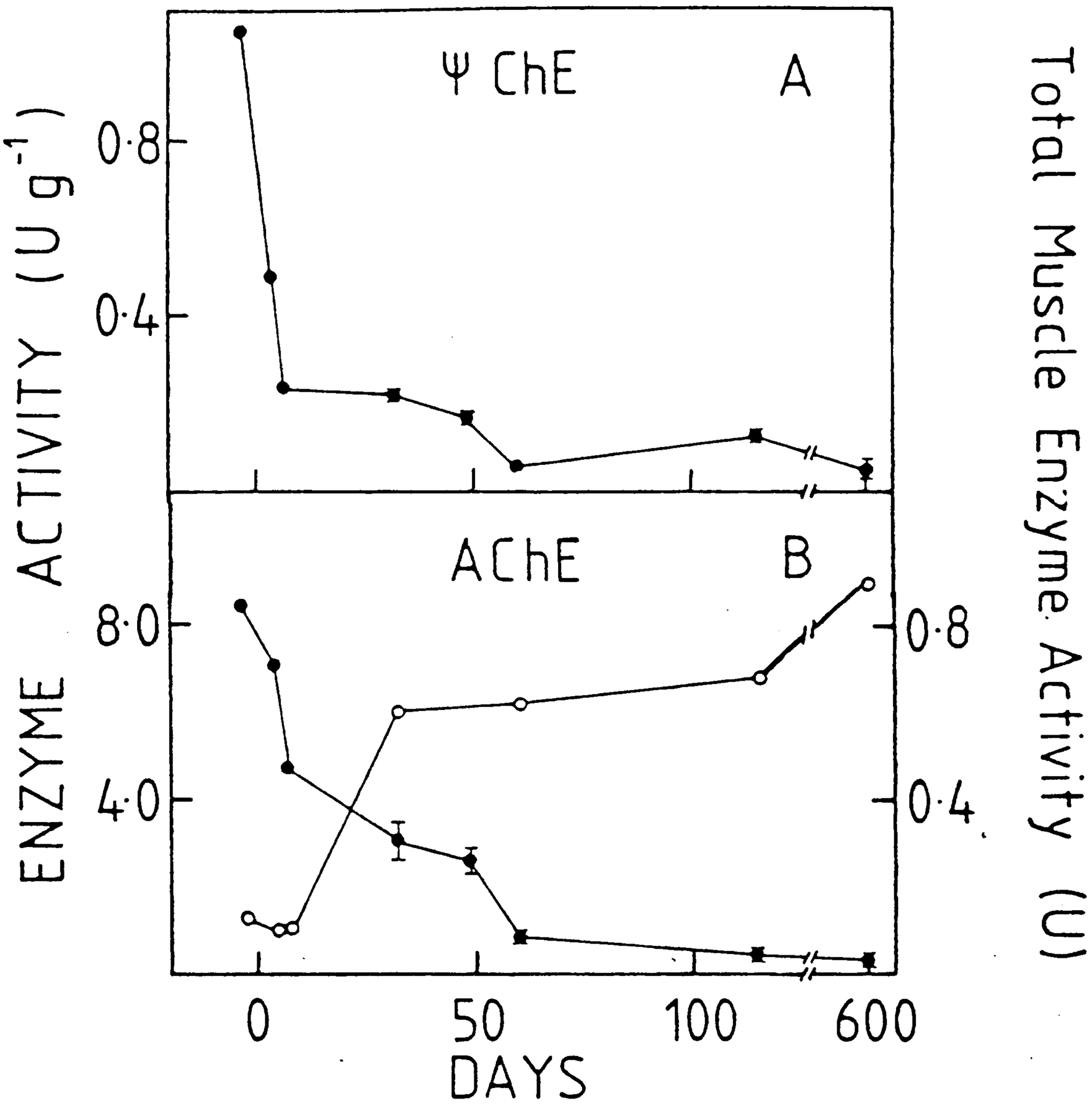


Figure 17. Concentrations of ψChE and AChE in the normal ALD muscle.

(A) ψChE; (B) AChE concentration per gram of muscle (●——●) or total enzyme per pair of ALD muscle (o——o).

The point to the left of day 0 represents d18 in ovo. Points up to d7 ex ovo represent a pool of muscle samples from 4 birds; at later ages the mean activity for 2-5 birds, with bars for S.E.M., is shown.

TABLE 14

LEVELS OF AChE AND ψChE IN DEVELOPING NORMAL AND DYSTROPHIC ALD MUSCLES

Age (d)		AChE (U/g)[a]		ψChE (U/g)[a]	
		Normal	Dystrophic	Normal	Dystrophic
4[b]		7.06	7.16	0.485	0.384
	n	4	4	4	4
7[b]		4.71	4.83	0.231	0.231
	n	4	4	4	4
32		2.22	2.26	0.152	0.148
	S.E.M.	±0.31	±0.21	±0.009	±0.009
	n	4	4	4	4
60		0.855	0.882	0.124	0.079
	S.E.M.	±0.106	±0.116	±0.025	±0.010
	n	4	3	4	3
115		0.439	0.462	0.129	0.189
	S.E.M.	±0.067	±0.032	±0.069	±0.009
	n	2	5	2	5
600		0.328	0.506	0.051	0.142
	S.E.M.	±0.078	±0.152	±0.023	±0.069
	n	2	2	2	2

[a]Values represent the mean (± S.E.) of the total enzyme activity per gram wet weight of muscle for n chickens tested.

[b]Values represent the automatic mean of the activity in muscles from n chickens since muscles at this age were pooled before extraction due to their small size.

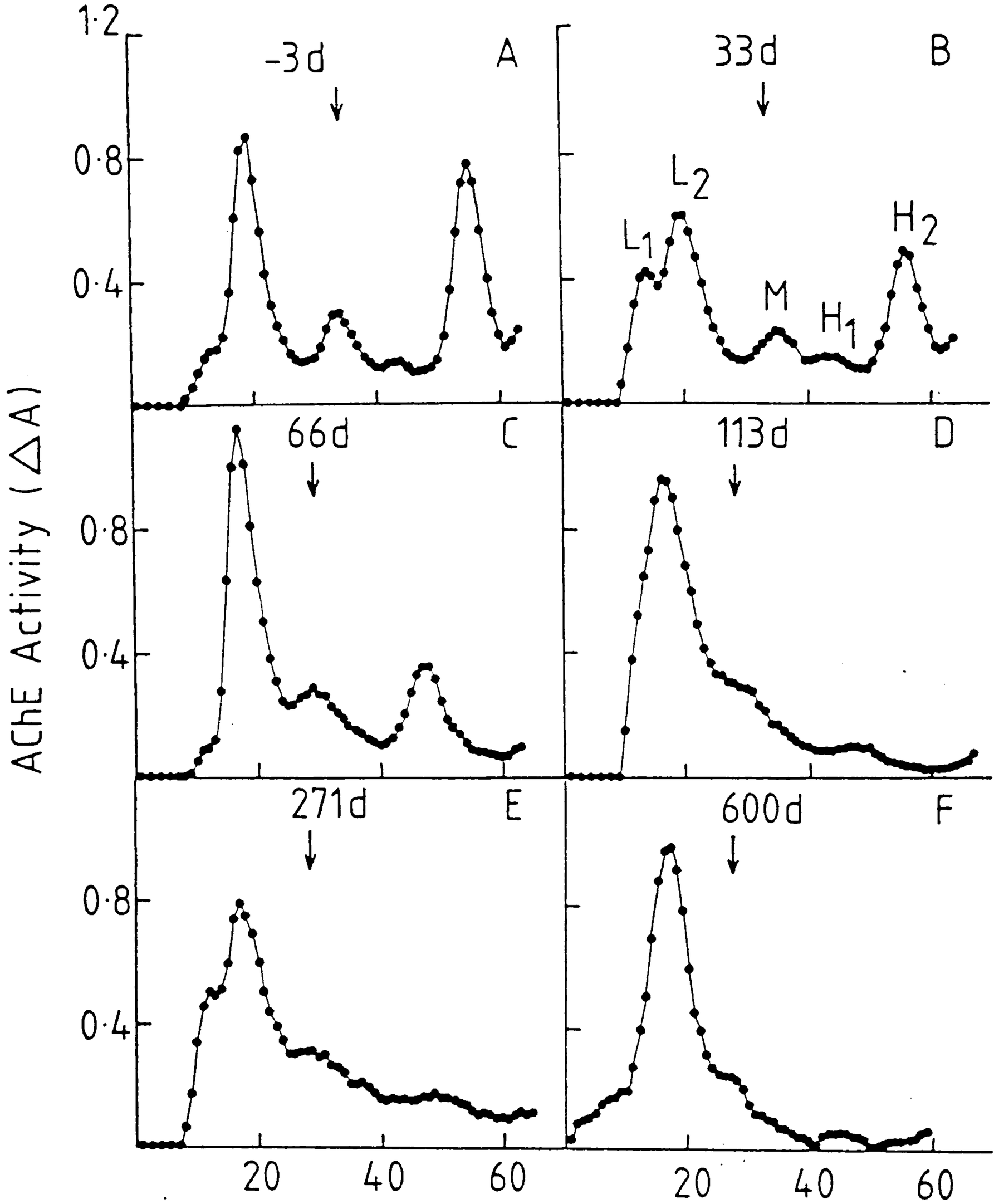


Figure 18. The changing molecular forms of AChE in the normal ALD muscle during maturation: the disappearance of H_2 in older slow tonic muscle.

The age of the muscle is indicated on each sucrose gradient profile. The abbreviations above certain peaks are as defined for pectoral muscle in Table 4. The single muscle in (C) was from a perfused bird. The number of birds sampled was: C, 1; D, 1; E, 1; F, 2.

IF: A, 0.5; B, 2.36; C, 3.75; D, 22.0; E, 9.0; F, 16.0.

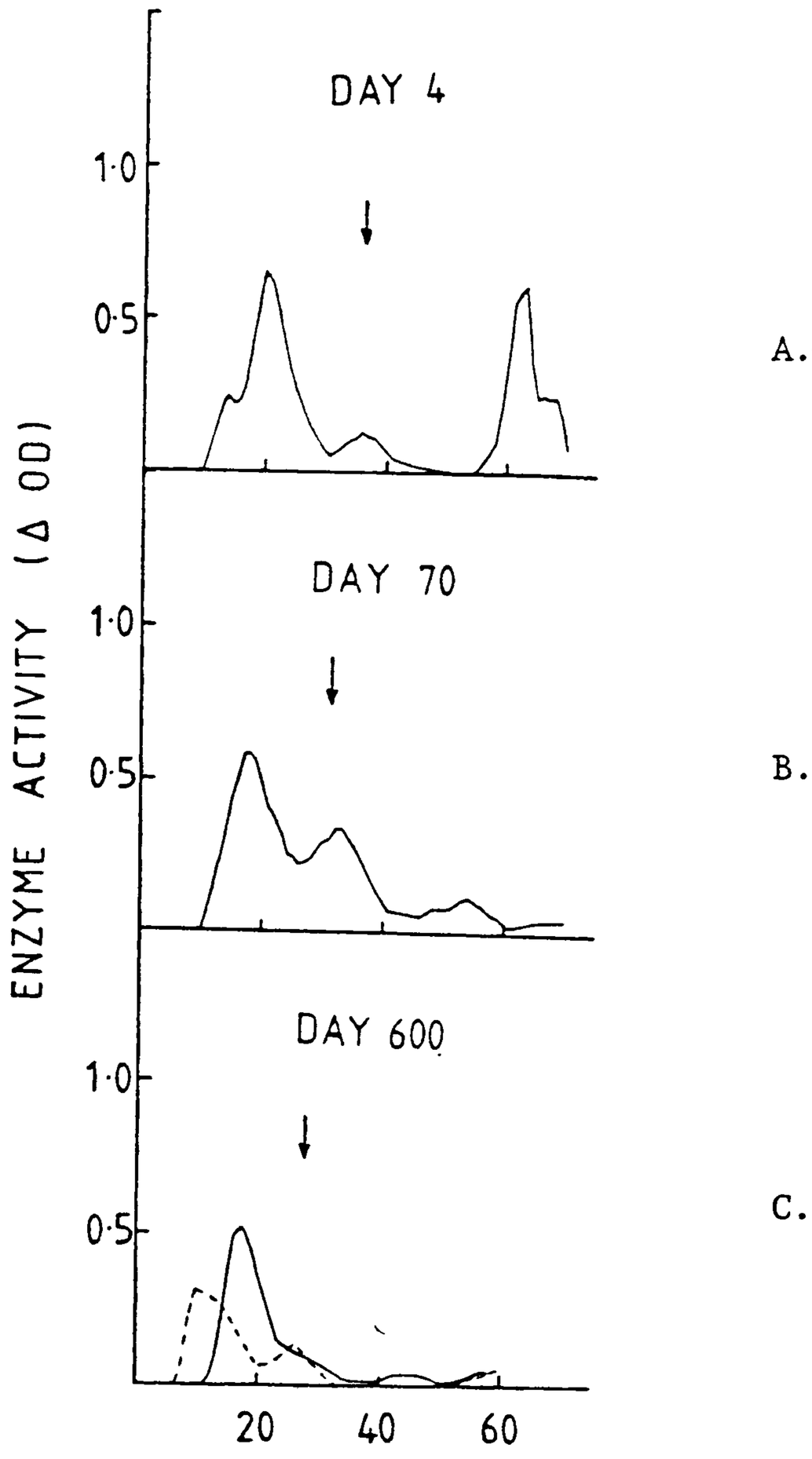


Figure 19. The molecular forms of AChE and ψChE in ALD muscle from dystrophic chickens.

The age of each muscle is indicated on each sucrose gradient profile. Solid lines represent AChE; the dashed line represents ψChE. Only 1 bird was sampled on d70 and 600.

IF: A, 0.75; B, 6.0; C-AChE, 9.0; C- ψChE, 15.0.

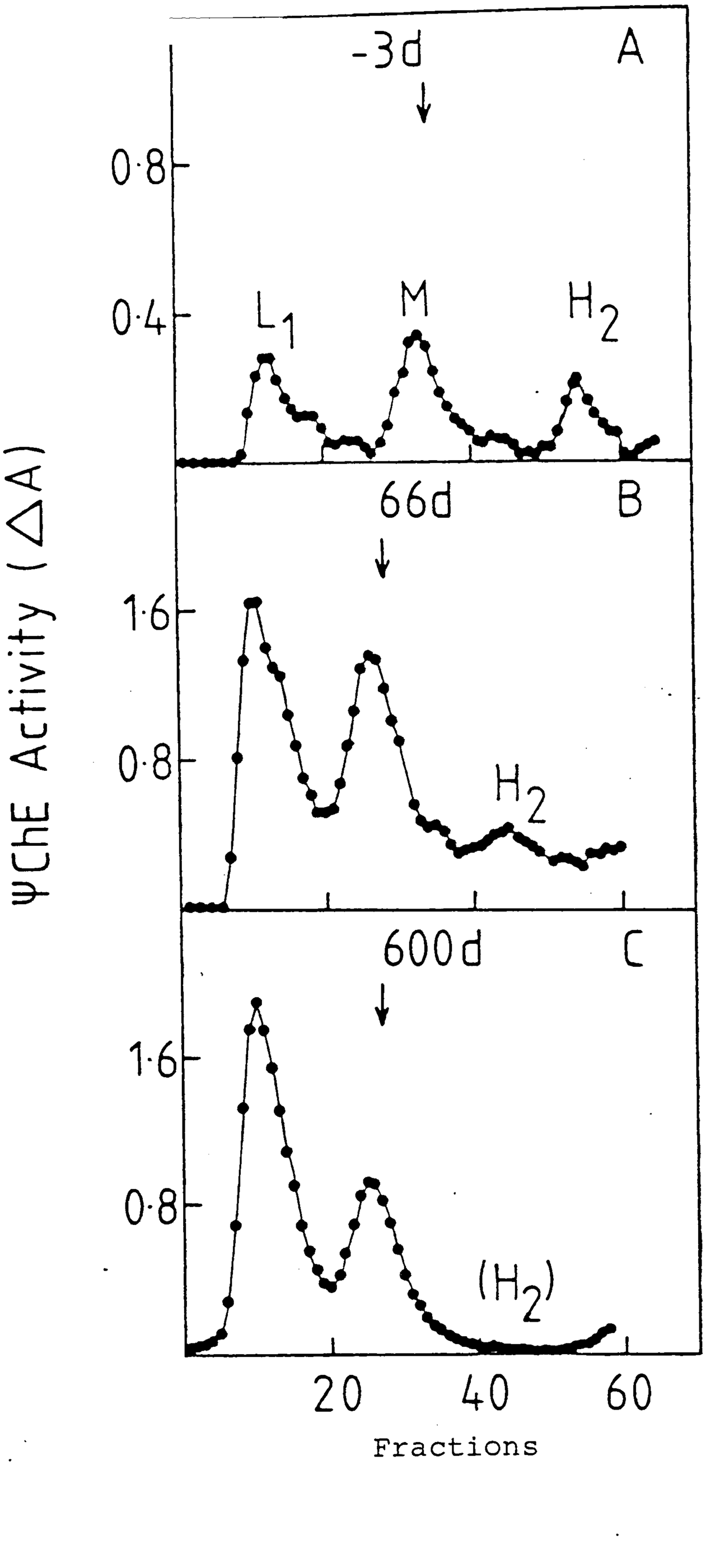


Figure 20. The changes in the molecular forms of ψChE in normal ALD muscle.

The age of the muscle is indicated on each sucrose gradient profile. The abbreviations above certain gradient peaks are as defined in Table 4. The muscle in (B) was perfused. The number of birds samples was: A, 4; B, 1; C, 2.
IF: A, 7.5; B, 3.75; C, 58.5.

ALD muscles from dystrophic chickens (Table 14). Although there is less ψChE than AChE, the concentration of ψChE is higher in the ALD than in normal fast twitch (pectoral and PLD) muscles.

The regulation of ψChE forms is similar to the control of AChE forms in the ALD muscle (Fig.20). All forms are produced in the embryonic muscle on d18, but there is less H_2 than L or M. There is a progressive diminution of the H_2 form and an increase in the proportion of L ψChE during further maturation ex ovo. No H_2 ψChE is detectable at d600. Regulation of ψChE forms is identical in ALD muscles from dystrophic chickens (e.g. d600, Fig.19).

5.2 Discussion

The ALD muscle is composed almost purely of red, slow tonic fibres. Its position is immediately anterior to the PLD. Originating on the ligament joining the first four thoracic vertebrae, it crosses over the back and inserts on the humerus. It is a postural muscle which is involved in supporting the wing. Slow tonic muscles are not reported to be affected by dystrophy in the chicken. Indeed, no alterations in the levels or forms of AChE or ψChE were detected in this study.

Mature, slow tonic muscles differ substantially from fast twitch muscles, as previously discussed (see 1.3). In the ALD, these differences are related to its specialization for the continuous, slow contraction needed to hold the wings in the normal resting position, as contrasted with the PLD whose function is to raise the wings, during flight or flapping, by fast and vigorous contractions. An important aspect of the study of AChE in the ALD is the character of its endplates. "En grappe" endplates are smaller and less transmitter is released from their nerve terminals upon stimulation than from

nerves at "en plaque" endplates. In keeping with these features, less AChE is also present at "en grappe" endplates on the ALD (see 1.3.1.1).

Regulation of the forms of AChE is also quite different to that in normal fast twitch muscles which results in the L_2 form being predominant in mature ALD. An indication of this difference in the regulation of enzyme forms is apparent as early as d18 in ovo, when the proportion of H_2 AChE is lower in the ALD than in fast twitch muscles. At this embryonic stage, endplates are clearly differentiated into "en grappe" and "en plaque" types: "en plaque" endplates appear as more intense finger-like stain, while "en grappe" endplates appear as thinner streaks of stain (see 1.3.2; also, Sisto Daneo & Filogamo, 1975). Thus, the distribution and molecular forms of AChE are differentiated by d18 in ovo in slow tonic and fast twitch muscles.

No differences between endplates of the ALD and PLD have been distinguished before d13-14 in ovo, since localized AChE is not detectable until synapses are well established, ca d14. Sisto Daneo & Filogamo did report an earlier difference between these two muscles: an increased proliferative activity of mononucleated cells at the future synaptic areas of myotubes of the PLD (before d11) and an absence of any such activity in the ALD muscle. They did not attribute any particular difference between the muscle fibre types to this activity. However, they, as well as Gordon et al (1974), did suggest that the development of different types of endplates between d11-14 is related to the increased and different patterns of electrical activity in the nerves to the two types of muscles at this stage. The two types of muscle fibres show further signs of differentiation by d16-17 in ovo regarding metabolic enzymes and contractility (see 1.3.2.3). Since the nerve influences the synthesis of the H_2

forms of AChE and ψChE in mature muscle (e.g. the effect of denervation, see 4.2.1.2), embryonic differentiation of the regulation of AChE and ψChE forms, as well as endplate differentation, may be due to the type of innervation which is established by d14. Thus, differences in enzyme regulation follow innervation and are apparent by d18 *in ovo*.

During further maturation of the ALD *ex ovo*, the H_2 forms of both AChE and ψChE decrease - gradually, at first, but more rapidly after d30. Thus, the change in the regulation of H_2 does not occur simultaneously with differentiation of the other fibre type characteristics which are established within the first several weeks *ex ovo*. The actual cause of the different regulation of enzyme forms in slow tonic and fast twitch muscles is not known. It could be determined by the pattern of contractile activity since, in addition to innervation, contractility has also been shown to regulate the synthesis and location of AChE in muscle cells *in vitro* and *in ovo* (see 4.2.1.2). More rapid contraction, as in fast twitch muscles, may promote the conversion of enzyme into tailed forms, while a lack of contraction (or slower contraction, as in the ALD) may not promote such a conversion process.

Since the ALD clearly has the capacity to synthesize tailed enzyme forms early in development, the disappearance of the tailed forms of AChE and ψChE might be due either to a repression or to a lack of stimulation of their synthesis in older muscle. The step during enzyme synthesis at which this regulation might be exerted could be the synthesis of the tail or of an enzyme or polypeptide necessary for the association of the tail and tetramers (as discussed in 4.2.1.2). The different pattern of contraction or nervous activity in the ALD might repress the step which may be necessary for the conversion of globular into tailed forms. However, since these

different patterns of activity are fully established within the first month ex ovo, it is unclear why, if these factors are involved in regulating AChE and ψChE, the H_2 forms disappear so slowly between 1 and 4 months in the ALD muscle.

The fact that no H_2 AChE exists in mature ALD muscle raises several questions. The first concerns the label of "endplate-specific form" which has been applied to H_2 AChE in striated muscles of the rat, human and chicken (Hall, 1973; Vigny et al, 1976b; Carson et al, 1979; Silman et al, 1979). The H_2 form has been shown to be more prevalent in endplate regions than elsewhere in certain muscles and has, therefore, been presumed to be the major form of functional importance at such endplates. Endplates of the ALD at d270 and d600, however, do stain for AChE although no significant amounts of H_2 AChE are present in the muscle. Thus, H_2 may be predominant at certain endplates, but it clearly is not the only form of functional significance that can occur at endplates since the tetramer (and possibly the dimer) must be located at ALD endplates in order to account for the histochemical staining of AChE seen in older muscle.

Second is the question of the mode of attachment of AChE at the endplate. McMahan et al (1978) assumed that the asymmetric form is attached to the synapse by the insertion of its tail into the collagen matrix of the basal lamina , leaving its tetrameric heads elevated above the membrane. The globular forms, however, are more likely to be integrated into the synaptic membrane since they lack a tail. Thus, they may lie flatter on the surface of the membrane than H_2. The tetrameric M form is presumed to be membrane-bound because of its low solubility in the absence of Triton X-100 and high levels of NaCl. It may be associated with internal membranes during the normal process of internal synthesis and transport to the cell surface.

The M form (10S) is known to be associated with the surface membrane in mouse neuroblastoma cells (Vigny et al, 1979b). Although its function on the surface of neuroblastoma cells may not be the same as on the surface of the muscle cell, it is evidence that the globular form can exist on surface membranes. It is most likely that the M form of AChE also occurs on the surface of muscle fibres at the ALD endplate, but how it is situated in the membrane is unknown.

The final question is: what is the functional significance of the predominance of the tailed form of AChE at "en plaque" endplates and of globular forms at "en grappe" endplates? There is much less AChE present at "en grappe" endplates, and the recovery time following stimulation is much slower in slow tonic than in fast twitch muscles of the frog (Magazanik et al, 1979). These facts indicate that there is a slower hydrolysis of ACh at "en grappe" endplates. Magazanik et al found that inhibition of AChE slows the recovery time in both slow tonic and fast twitch muscles. This effect is slightly greater at fast twitch endplates which led Magazanik et al to suggest that diffusion (across a larger synaptic gap) may play a bigger role in the removal of ACh from "en grappe" than from "en plaque" endplates.

Such differences may also be related to a different arrangement of AChE molecules around receptors at the two types of endplates. Barnard et al (1980) have proposed that one H_2 AChE molecule (i.e. 12 catalytic subunits on a tail or "stalk") is surrounded by ca 40 receptors at "en plaque" endplates of fast twitch muscles, enabling a fairly rapid removal of ACh from the receptors. At "en grappe" endplates the globular forms, probably lying flatter on the membrane, may not be accessible or mobile enough to allow for as rapid a removal of ACh from receptors as occurs at "en plaque" endplates. The tetramer or dimer forms (4 or 2 catalytic subunits, respectively)

located at "en grappe" endplates provide a less concentrated local packaging of AChE activity. The lack of junctional folds at these endplates further decreases the density of AChE, as compared to "en plaque" endplates where AChE covers the secondary, as well as the primary, junctional folds. The physical arrangement of globular forms of AChE at "en grappe" endplates may, therefore, not allow for as rapid a hydrolysis of ACh as at "en plaque" endplates. Since the function of slow tonic muscles does not require a fast recovery time, the regulation of AChE forms, like many other fibre type characteristics, may be adapted to muscle function. As with other embryonic properties which are lost during muscle fibre maturation, the H_2 form of AChE may disappear from the ALD during development because it is not needed to enable the pattern of contraction required in slow tonic muscles.

ψChE is regulated in the same way as AChE in the ALD muscle, providing further evidence for the parallel regulation of these two enzymes. The fact that ψChE is more prominent in slow tonic, than in fast twitch, muscle may imply that its function in muscle is to assist AChE in hydrolyzing ACh in the event of any abnormal occurence of high ACh levels, as suggested by Silver (1974). If the arrangement of AChE is less efficient at "en grappe" endplates, as has been proposed here, then the need for such an auxillary enzyme would be greater in slow muscles than at "en plaque" endplates of fast twitch muscles where AChE may be organized for more efficient hydrolysis of ACh.

5.3 Summary

The slow tonic ALD muscle synthesizes the same forms of AChE and ψChE as those produced by fast twitch muscles, but its regulation of the synthesis of these forms is different. This difference is apparent by the end of embryonic development when other fibre type characteristics have begun to differentiate. At this

stage the L form is more prevalent and there is less H_2 than in fast twitch muscles. A progressive decrease in the proportion of H_2 AChE and ψChE occurs throughout further development ex ovo until virtually none is detectable after four months. Therefore, globular forms of AChE exist at "en grappe" endplates which must be attached without a collagen tail. The arrangement of tail-less forms at synapses may provide for less efficient hydrolysis of ACh. The predominance of M and L forms of AChE in the ALD is, therefore, proposed here as being correlated with the functional character of the muscle (i.e. slow contraction). The tailed forms of AChE, on the other hand, are suggested as being associated with more rapid ACh hydrolysis as required at "en plaque" endplates in fast twitch fibres. The greater prevalence of ψChE in slow tonic, as opposed to fast twitch, muscle may be related to this proposed scheme; an auxi l iary enzyme would be more essential at "en grappe" than at "en plaque" endplates should any occasional excess of ACh occur.

CHAPTER 6

PLASMA

6.1 Introduction

The forms of AChE and ψChE which predominate in the plasma of the chicken are globular ones. They have similar sedimentation coefficients to those which are present in muscle (Table 15). In certain plasma samples, there is also an additional light form of AChE (7.4S) and heavy form of both AChE and ψChE (ca 15.5S). Three plasma forms of AChE and ψChE, similar to L_1, L_2 and M illustrated here, have also been reported by Rotundo & Fambrough (1979) since this study began. In contrast to their report, a clear difference in the sizes of the corresponding forms of AChE and ψChE has been distinguished in this study. The forms of ψChE in plasma, as in muscle, are smaller than the AChE forms.

L_1, L_2 and M correspond to the forms in muscle which have monomeric, dimeric and tetrameric structures. The heavy forms were only occasionally detected in plasma, usually from dystrophic chickens with high enzyme activity. These may represent multi-tetrameric structures, as do the heavy forms in muscle, which lack most of the tail.

The chicken is known to differ from many other species in not having AChE on its red blood cells (Zajicek et al, 1954; Silver, 1974). The question of AChE activity on the chick red blood cells was re-investigated in order to confirm that such cells are not a source of plasma AChE especially in the dystrophic chicken (Table 16). Whole red cells from normal chickens, suspended in isotonic saline, showed no detectable AChE activity. Red cell ghosts were prepared by lysing the cells (previously separated from leucocytes and washed with saline) in water and by sedimentation at 10,000 x g for three minutes. These ghosts, or a 1% Triton X-100 extract, showed low but detectable AChE activity in the presence of isoOMPA (<4% that

measured on rat red blood cell ghosts). No ψChE was detected. Although the level of AChE activity in normal plasma is low, ψChE levels are high. Since no ψChE activity was detected in red cells or ghosts, it was concluded that any plasma enzyme had been successfully washed off the red cells. The results show that there is a very small amount of AChE on chick red blood cells. When a 1% Triton X-100 extract of red blood cells from a 4 week old dystrophic chicken was assayed a similar low level of AChE, but no ψChE, was detected. Therefore, the increased AChE activity in plasma of dystrophic chickens is not due to any increased AChE in red blood cells.

6.2 Acetylcholinesterase

6.2.1 Results

AChE increases in embryonic plasma from d14 to d19 but decreases sharply during the first week *ex ovo* in plasma from both normal and dystrophic chickens. AChE decreases further in normal plasma to reach very low levels (5-10% of total plasma cholinesterase activity) by several weeks *ex ovo*; levels remain low throughout further maturation (Fig.21A). AChE in plasma from dystrophic chickens follows a similar pattern up to approximately three weeks *ex ovo*. However, AChE levels are distinctly lower than normal during this early period of development. After three weeks, AChE activity increases, peaking around four months, and then declines to nearly normal levels between one and two years.

The major form (*ca* 60-80%) of AChE in plasma is M. L_2 is the main light form, but small amounts of L_1 are also present (Fig.22, Table 17). The proportions of the various AChE forms are similar in plasma from embryonic and adult normal chickens. In plasma from dystrophic chickens there is a progressive increase of AChE:

TABLE 15

SEDIMENTATION COEFFICIENTS OF THE MOLECULAR FORMS OF AChE AND ψChE IN CHICKEN PLASMA AND MUSCLE

	L_1	L_2	L_3
AChE			
Plasma	4.6 ± 0.1	6.6 ± 0.1	7.4 ± 0.1
n	12	13	10
Muscle	4.7 ± 0.1	6.6 ± 0.1	
ψChE			
Plasma	4.4 ± 0.1	6.0 ± 0.1	--
n	16	16	
Muscle	4.0 ± 0.1	5.9 ± 0.1	
Suggested assignment[b]	Monomer	Dimer	?

Values represent the mean sedimentation coefficient ($s_{20,w}$) in Svedberg units and its S.E. for n determinations. The values of $s_{20,w}$ for horse liver alcohol dehydrogenase (4.8S), beef liver catalase (11.4S), and E. coli β-galactosidase (16S) and the positions of these standards in the sucrose gradients were used in determining the sedimentation coefficients reported.

[a] H_{1A} and H_{1B} were not regularly detected but were occasionally found in dystrophic plasma, particularly from older birds with high enzyme activity. Their presence was often obscured by the high peak of activity at the M region.

[b] Assignment of subunit structure, assuming that the conclusions of Bon et al (1979) for AChE forms in muscle apply.

TABLE 15 -- Continued

M	H_{1A}[a]	H_{1B}[a]
11.9 ± 0.1 20	13.7 ± 0.1 9	15.7 ± 0.1 5
11.1 ± 0.1	14.7 ± 0.2	
11.1 ± 0.1 16	13.8 ± 0.1 6	15.5 ± 0.1 5
10.5 ± 0.1	14.1 ± 0.1	
Tetramer (no tail)	?	?

TABLE 16

AChE AND ψChE ACTIVITY IN

RED BLOOD CELLS AND FAT

	AChE	ψChE
Red blood cell ghosts[a]		
	ΔOD/min/ml	ΔOD/min/ml
Chick		
Normal	0.3	0
Dystrophic	0.5	0
Rat	7.4	
Body fat[b]		
	U/g	U/g
	0.185	0.0

[a] Saline-washed ghosts from 4 week old chickens were resuspended in an aliquot of 1% Triton X-100 which equalled the original blood sample volume. 0.04ml was assayed in 1.0ml Ellman reagent.

[b] Tissue derived from the back of a 12 week old dystrophic chicken.

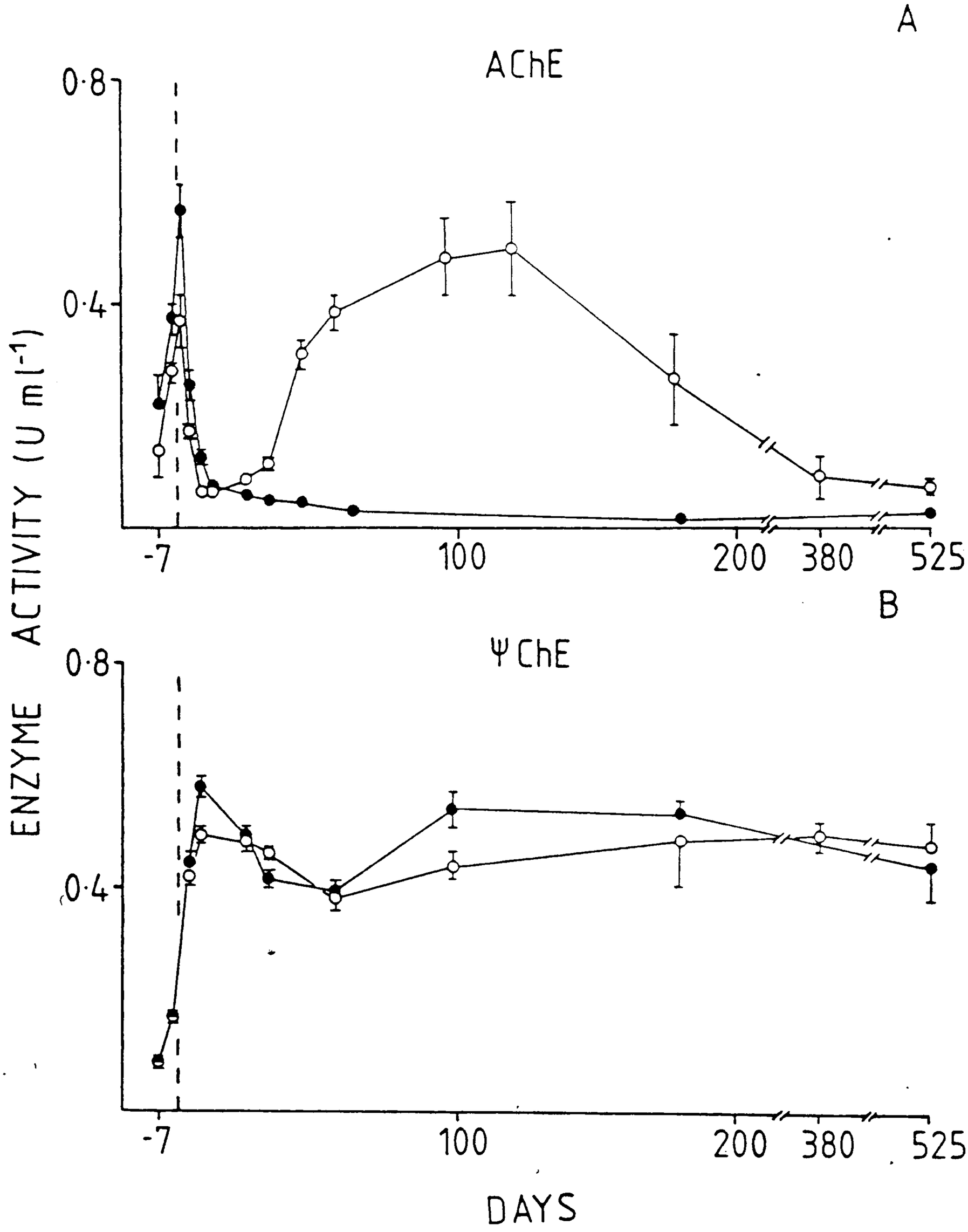


Figure 21. Changes in AChE and ψChE concentrations in plasma of developing normal and dystrophic chickens.

Normal (●——●); dystrophic (o——o). The broken vertical line represents the day of hatching. Each point is the mean, representing n (11-34) chickens except: at d14 in ovo (d-7) n (normal) = 6, n (dystrophic) = 3; at d100 n (normal) = 7; at d389 n (dystrophic) = 4, at d525 n (normal) = 4, and n (dystrophic)= 7. Bars represent S.E.M.

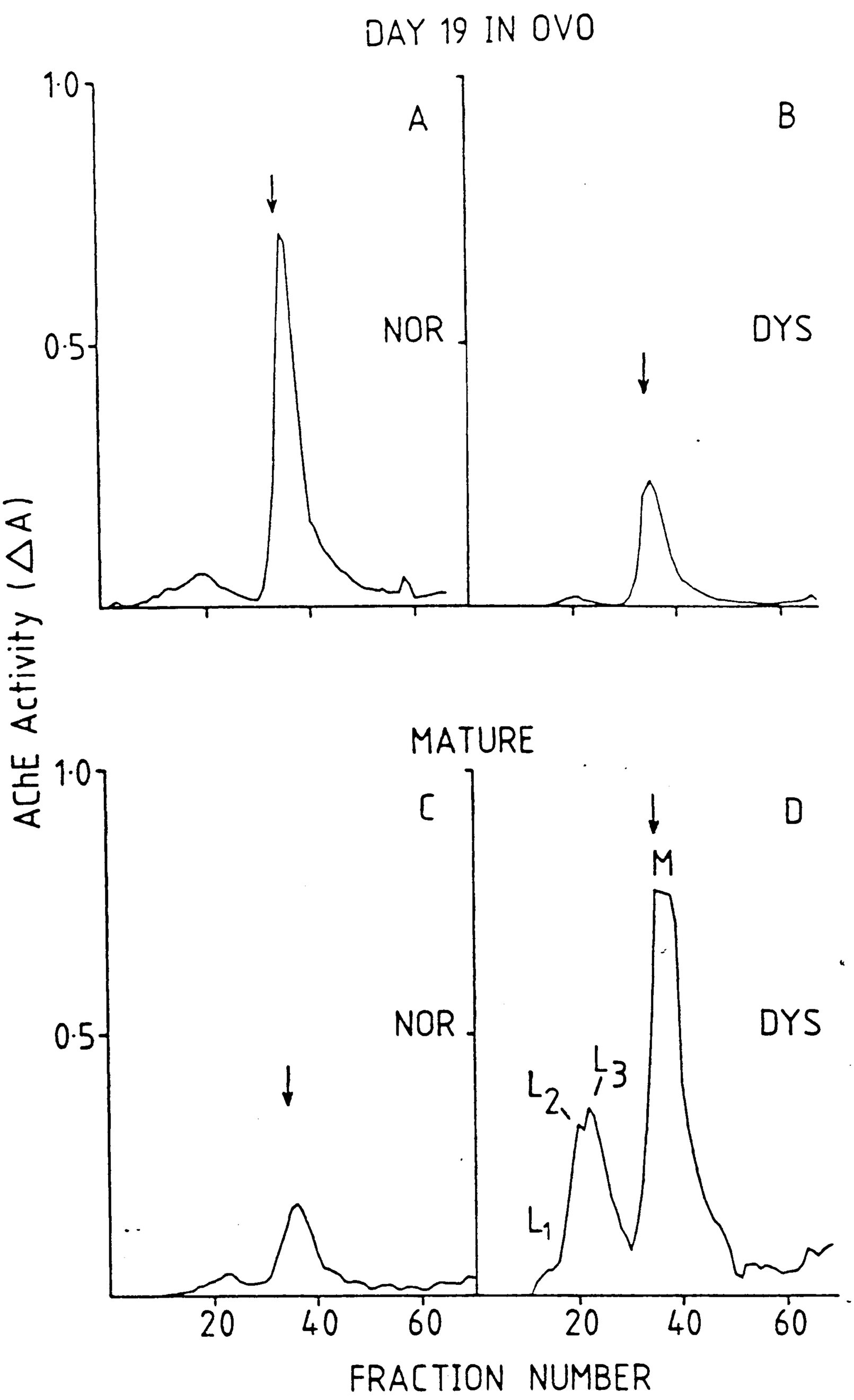


Figure 22. The molecular forms of AChE in plasma from normal and dystrophic chickens in ovo and ex ovo.

Plasma in (C) and (D) were obtained from birds aged d50. The abbreviations above peaks in (D) are defined in Table 15.

TABLE 17

CHANGES IN THE MOLECULAR FORMS OF AChE IN PLASMA OF THE DEVELOPING NORMAL AND DYSTROPHIC CHICKEN

		Day 19 in ovo	Day 4	Day 22	Day 45	Day 96
L_2						
% of total AChE activity	N	3	9	23	15	18
	D	6	7	22	36	20
U/l of L_2	N	11	23	12	7	7
	D	17	12	14	112	97
% change in L_2 activity from normal		+54	-48	+17	+1500	+1290
M						
% of total AChE activity	N	78	77	67	78	57
	D	81	79	65	51	63
U/l of M	N	291	196	34	35	22
	D	224	135	40	159	304
% change in M activity from normal		-23	-31	+18	+350	+1520
Ratio L_2/M (U/l)	D	0.08	0.09	0.35	0.70	0.32
	N	0.04	0.12	0.35	0.20	0.32

The terms L_2 and M are defined in Table 15 except that here the amounts of the L_3 form are included in the amounts listed for L_2 because the two forms often could not be differentiated. Only the two areas, M and (L_2+L_3) of AChE were quantified since the amount of activity present in other forms was very small. N, normal; D, dystrophic.

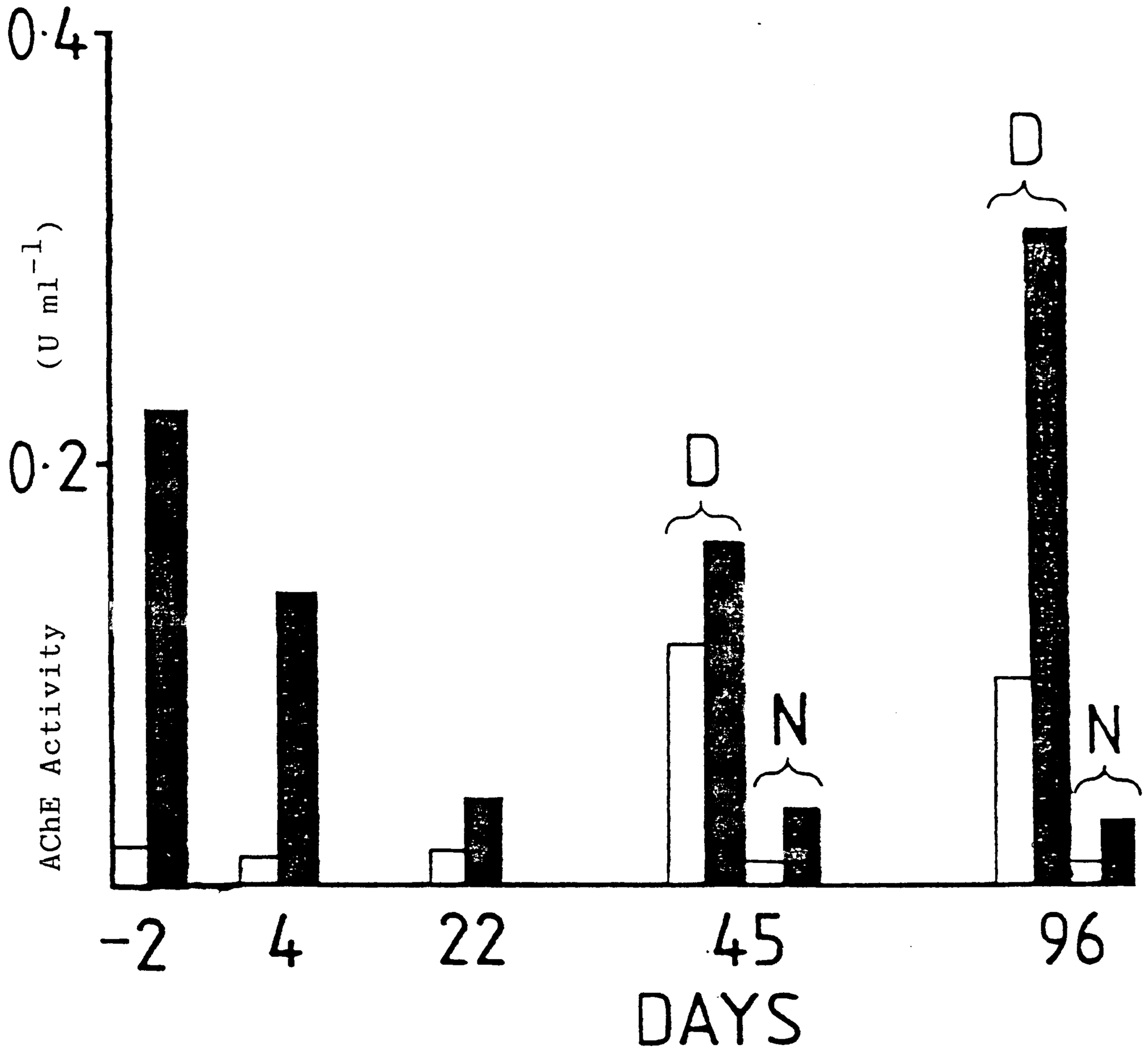


Figure 23. A bar graph illustrating the changing levels of L_2 and M forms of AChE in plasma from dystrophic chickens during development and from normal chickens on d45 and d96.

L_2, open bars; M, solid bars. D, dystrophic; N, normal. The levels of L_2 and M in normal plasma on days -2, 4 and 22 are similar to those shown for plasma from dystrophic birds at those ages.

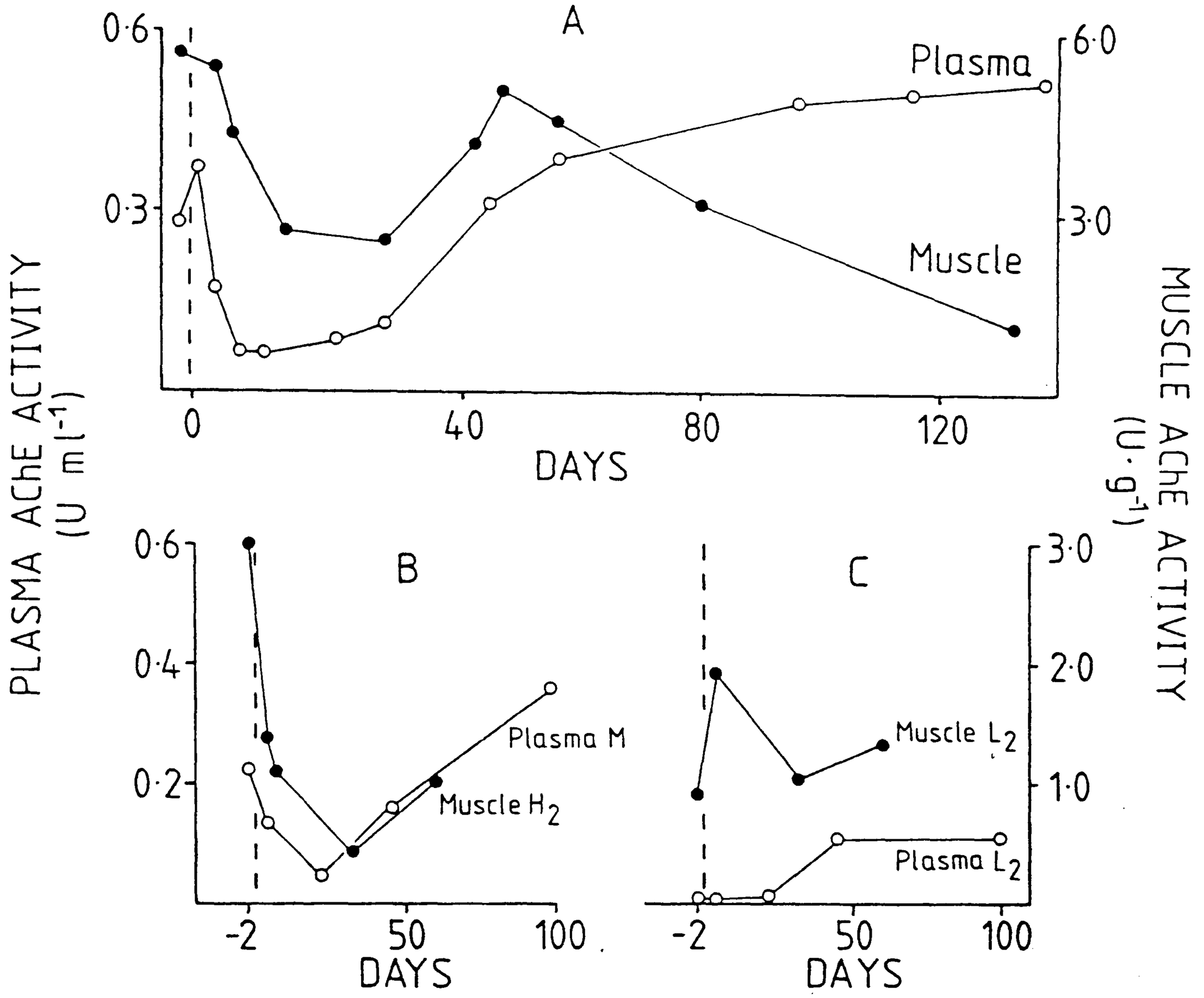


Figure 24. A comparison of the changing levels of AChE during development in the pectoral muscle and plasma of dystrophic chickens.

Plasma (o——o); pectoral muscle (•——•). (A) the concentration of AChE (all forms); (B) similar changes in the concentrations of plasma M (o——o) and muscle H_2 (•——•); (C) changes in the concentrations of L_2.

first, in the amount of L_2, as on d45, and, later, in the amount of M (Fig.23). This is illustrated by the changing ratio of L_2:M which is markedly increased on d45 (0.71) compared to the ratio at earlier or later ages (ca 0.3).

A comparison of the increased levels of AChE in the plasma and pectoral muscle of the dystrophic chicken is shown in Fig.24. The increased levels of AChE which are found in the muscle between d30-60 are parallelled by the elevation of AChE which occurs in plasma between d30 and d120. In plasma, however, the concentration of AChE continues to increase after d120, whereas it decreases in muscle after d60. The changing concentrations of the different forms of AChE in plasma and muscle show a particular correlation of the M plasma form with the H_2 form in muscle during early stages of dystrophy.

6.2.2 Discussion

AChE accounts for a very small proportion of the total cholinesterase activity in the plasma of mature normal chickens and is not apparently derived from red blood cells. Considerably elevated levels of AChE, however, occur in plasma from embryos and very young chickens. Such instances of high plasma AChE activity, along with the fact that cultured muscle cells release AChE, led Wilson et al (1973b) to suggest that muscle is the source of excess AChE in the plasma of dystrophic and young normal chickens. No great increase of AChE has been found in red blood cells (in this study) or in nerves (Di Giamberardino et al, 1979) of dystrophic chickens, further indicating that muscle is the most likely source of plasma AChE in the dystrophic chicken.

ACh receptors become concentrated at the synaptic area of muscle fibres during embryonic development. Betz et al (1977) proposed that excess extrajunctional receptors are eliminated from

the cell surface by degradation. A similar process may likewise eliminate AChE from the extrajunctional area of embryonic muscle fibres. If this is achieved by a degradation of the extracellular heavy forms, it could explain the temporary occurrence of high AChE levels in the plasma of embryos and young chicks.

It is particularly interesting that the levels of AChE during the early developmental period were found in this study to be lower than normal in both plasma and fast twitch muscles of dystrophic chickens. Such a correlation further supports the idea that muscle is the source of AChE activity in plasma. It also indicates that an early difference in dystrophic chickens is related to a lower than normal synthesis of AChE in muscle.

Muscle cells release AChE when grown in tissue culture, but the major form released is 9S (Rotundo & Fambrough, 1979) -- a form not detected *in vivo*. Small amounts of a 15S form were also detected in the culture supernatant *in vitro* which could correspond to the 15S form detected in this study of AChE in plasma. Rotundo & Fambrough found that collagenase had no effect on either of the secreted forms. The forms released from muscle cells *in vitro* therefore lack a tail. This is also assumed to be the case for the forms detected here in plasma since they are soluble, rather than membrane-bound, and correspond (according to sedimentation coefficients) to globular muscle forms or the non-tailed 15S form detected by Rotundo & Fambrough.

The active secretion of a 9S form in vitro may not be comparable to the process by which 11S AChE arrives in the plasma. A prominent difference between the two cell systems is that no tailed forms were produced by muscle cells *in vitro*. Most of the muscle enzyme *in vivo* exists externally, and, in fast twitch muscles, as the tailed form. If H_2 is degraded outside the cell, 11S tetramers could be released and

removed by the plasma. Trypsin has been shown to release 11S subunits from the tailed forms of Electrophorus AChE (Bon et al, 1979). Proteolysis, in general, causes the breakdown of larger AChE forms into smaller ones (Silman et al, 1978).

An indication that plasma AChE might arise by a degradative process from muscle AChE is the fact that the M forms of AChE in these two tissues have slightly different sedimentation coefficients (Table 15). This could indicate that the M form has a different conformation when soluble in the plasma than when membrane-bound in muscle. Alternatively, it may indicate that the M form in plasma is derived by a cleavage of tetramers from the H_2 form. This process may result in a fragment of the tail being left attached to the tetramer. Thus, the degradative M form in plasma would be slightly larger than the M form produced during the synthesis of AChE in muscle. Such a process could account for the small amounts of AChE in normal plasma. It could also account for the higher levels of enzyme in plasma from dystrophic chickens since there is an increased amount of AChE available for degradation on the surface of dystrophic fibres. In addition, higher levels of muscle proteases have been found in dystrophic muscle (Iodice et al, 1972) which might assist in the degradative process.

The membranes of dystrophic muscle fibres are presumably more permeable or "leaky" than normal (see 1.4.2.3), thus allowing soluble muscle proteins to escape into the plasma. If excess AChE arrives in the plasma of dystrophic chickens simply by the process of leakage, the proportions of the forms present in plasma should reflect the proportions of the soluble forms which are overproduced in the muscle, namely L_2. L_2 AChE does increase more than M early in the dystrophic process (e.g. on d45, Fig.23). This may reflect an increase in membrane permeability or cell destruction during the initial stage of muscle

hypertrophy which enables enzyme leakage. The relative proportions of L_2 and M in plasma at later ages, however, do not mirror their ratios in the dystrophic muscle. Instead, these forms are present in approximately the same proportions as in normal plasma. A correlation of the increasing concentrations of the H_2 form in muscle with the M form in plasma during the early stages of dystrophy further supports the view that plasma AChE is derived by degradation of larger forms produced in muscle. It is therefore likely that excess plasma AChE in the dystrophic chicken is the result of an increased degradative process.

The levels of AChE do not change in exactly the same way in dystrophic muscle and plasma after d60; they decrease sooner in muscle than in plasma. Several explanations could account for this difference. First, the overproduction of enzyme in muscle might slow down, while excess levels persist in the plasma. It was shown in 4.2.1.1, however, that dystrophic fibres have approximately 45 fold more AChE than normal even on d600. The apparent decrease in enzyme concentration in older muscle is due to a reduced percentage of muscle fibres, not to a reduced synthesis of enzyme. Thus, the concentration of AChE per gram of muscle decreases during the phase of muscle atrophy, but its overproduction per gram of muscle fibre is not diminished.

Second, the increased enzyme levels which occur in plasma between d60 and d120 may be due to a greater release of enzyme from muscle. The initial period of dystrophic muscle hypertrophy gives way to the processes of atrophy and muscle cell degradation after ca d60. The functional ability of the dystrophic pectoral muscle also worsens markedly between d40 and d60. Thus a massive release of AChE into the plasma may result from the disintegration of muscle cells.

Third, blood volume increases with age in relation to body size. The dystrophic muscle, on the other hand, atrophies with age. Even if the total production and release of AChE were constant in dystrophic muscle, increasing blood volume would dilute its concentration in plasma at older ages, assuming a constant rate of enzyme degradation. It is probably this final factor, along with the reduced number of muscle fibres present, which causes the nearly normal AChE levels detected here in older dystrophic chickens. An understanding of the fluctuation in the levels of plasma AChE during the course of dystrophy is therefore important when using this parameter as a marker for the dystrophic condition.

6.3 ψChE

6.3.1 Results

The levels of ψChE in plasma from normal and dystrophic chickens are similar at all ages (Fig.21B). ψChE increases dramatically between d19 *in ovo* and d4 *ex ovo* and is maintained at constant, high levels throughout further development. ψChE accounts for 90-95% of the cholinesterase activity in normal plasma. Its percentage varies in plasma from dystrophic chickens due to fluctuations in the levels of AChE, but the absolute concentrations of ψChE are similar to those in normal chickens.

The forms of ψChE present in plasma are also identical in normal and dystrophic chickens (Fig.25; Table 18). The M form accounts for 60-70% of the enzyme activity. L_1 and L_2 account for 10-20% each. The proportions of the forms are similar in embryonic and adult plasma, except that there is slightly more of the light forms *in ovo*.

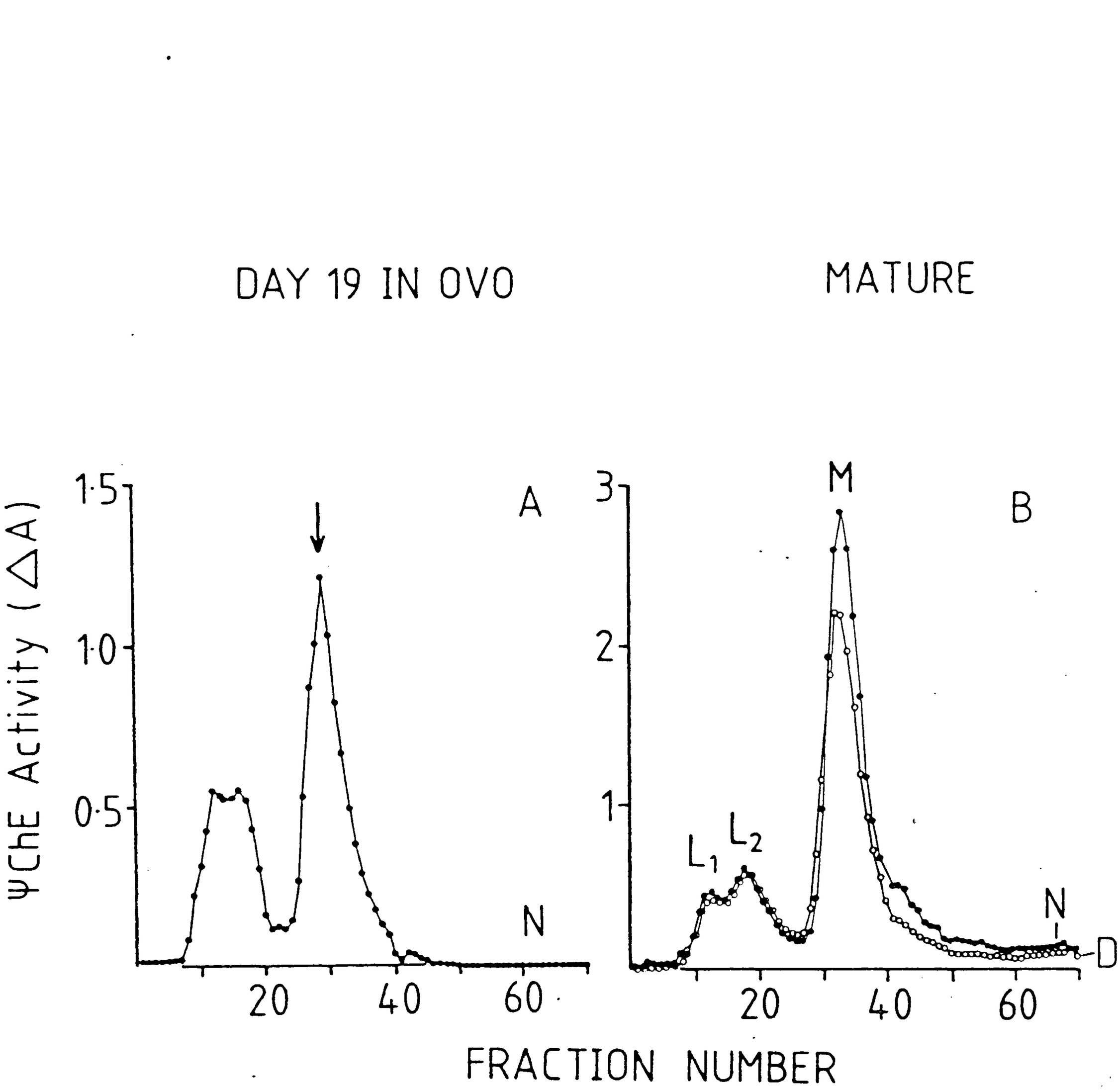


Figure 25. The molecular forms of ψChE in plasma from normal and dystrophic chickens in ovo and ex ovo.

Normal (●——●); dystrophic (o——o). Mature plasma is from birds aged d50. The abbreviations above the peaks (B) are defined in Table 15.

TABLE 18

CHANGES IN THE MOLECULAR FORMS OF ψChE IN PLASMA OF THE DEVELOPING NORMAL AND DYSTROPHIC CHICKEN

		Day 19 in ovo	Day 4	Day 22	Day 45	Day 96
L_1						
% of total ψChE	N	16	15	11	7	10
	D	21	19	14	10	13
U/l	N	28	67	55	31	54
	D	34	80	69	42	57
L_2						
% of total ψChE	N	17	20	18	18	17
	D	21	19	17	14	15
U/l	N	29	89	89	81	92
	D	34	80	84	59	66
M						
% of total ψChE	N	59	58	68	68	59
	D	53	54	67	70	63
U/l	N	101	258	337	305	320
	D	86	226	332	296	278

The terms L_1 and M are defined in Table 15. L_2 is defined in Table 17. N, normal; D, dystrophic.

6.3.2 Discussion

ψChE occurs in the plasma of the chicken and in other species in multiple molecular forms (see 1.2.3). This study has shown that the forms of ψChE, like AChE, which are found in plasma of the chicken are the globular ones, with the M form predominant. Like many plasma proteins, plasma ψChE is known to be synthesized in the liver (Svensmark, 1963). A regulatory mechanism may, therefore, exist in the liver which controls normal levels of ψChE in the plasma. Such a mechanism is suggested by the fact that ψChE concentration is relatively constant in the plasma throughout maturation regardless of blood volume or body size. Also, the establishment of adult ψChE levels at the time of hatching correlates with the development of liver function. This is in contrast to the earlier rise *in ovo* of plasma AChE which correlates better with earlier development and increase in AChE content in muscle.

It is curious that ψChE, like AChE, is quite elevated in dystrophic muscle, yet no excess ψChE appears in the plasma. This may be due to the regulatory function of the liver in removing excess ψChE. Many liver-produced proteins have been shown to carry sialic acid markers which prevent their recognition and destruction by liver hepatocytes (Svensmark, 1963; Ashwell & Morell, 1974). If ψChE from muscle does not have such a sialic acid marker, any excess ψChE released into the plasma could thus be recognized as "foreign" and eliminated by the liver. AChE from *Electrophorus*, on the other hand, has been shown to have sialic acid residues on the tailed form and a smaller number of such residues on the globular form (Rieger *et al*, 1973b). If AChE from chicken muscle also contains sialic acid residues, this might explain its persistence in the plasma of dystrophic chickens, since it would not then be recognized and degraded in the liver.

An alternative explanation to account for normal ψChE levels in plasma from dystrophic chickens is that ψChE is not released from dystrophic muscle. This was suggested in 4.2.2.2 as a possible explanation for the continued presence of the dystrophic pattern of ψChE forms in very old muscle, as well as the non-parallel regulation of AChE and ψChE levels in muscles of dystrophic chickens after d90. However, it seems unlikely that ψChE is not released from dystrophic muscle. First, there is an increased leakage of so many other muscle enzymes from dystrophic fibres into the plasma. Second, the integrity of the membrane is presumably abnormal. Third, ψChE is generally considered to be more soluble than AChE. Why, then, should ψChE, in particular, not leak out also?

It is, therefore, proposed that a regulatory mechanism functions normally in dystrophic chickens which maintains constant ψChE levels in the plasma if excess ψChE is released from muscle. This would resolve the discrepancy of finding abnormal AChE levels and normal ψChE levels in the plasma of dystrophic chickens. It is felt that the apparent non-parallel regulation of the synthesis of these enzymes in dystrophic muscle may, in fact, be due to the synthesis of ψChE in fat or connective tissue, rather than non-parallel synthesis or release of the two enzymes in muscle fibres (see 4.2.2.2). Connective tissue does not release ψChE when grown in tissue culture; if it did release ψChE *in vivo* the enzyme would probably be eliminated by the liver and so would not alter plasma levels.

6.4 Summary

The forms of AChE and ψChE which are found in blood plasma are the globular ones (L and M). AChE is normally present in plasma in very low levels; it may be derived from AChE in muscle and arrive

in the plasma by degradation of synaptic AChE. AChE concentration in plasma from dystrophic chickens is directly related to the enzyme levels in dystrophic muscle. Lower than normal levels occur in both tissues in ovo; excess enzyme appears in the plasma by three weeks ex ovo following earlier increases of AChE in dystrophic muscles. Further elevations of plasma AChE follow the changing enzyme levels in muscle until nearly normal levels are restored in very old dystrophic chickens. The proportions of the various AChE forms are similar in plasma from normal and dystrophic chickens, with M being predominant. This indicates that the plasma enzyme in both cases is derived by the same process, namely by degradation rather than by leakage of muscle AChE.

ψChE accounts for most (ca 95%) of the cholinesterase activity in normal plasma; the M form is the major form present. The levels and forms of ψChE are unaltered in plasma from dystrophic chickens. Three possible explanations are proposed to explain this: 1) ψChE is not released from dystrophic muscle; 2) muscle ψChE is released into plasma but is metabolized and eliminated by the liver; or 3) ψChE is produced by a non-muscle cell in dystrophic muscle which does not release the enzyme into the blood.

CHAPTER 7

THE EFFECT OF CHEMOTHERAPY ON AChE AND ψChE

7.1 Introduction

The levels of several muscle enzymes which appear in the plasma of dystrophic chickens (e.g. CPK, LDH, PK and muscle AChE) have previously been used to assess the effects of various chemotherapeutic drugs (Barnard et al, 1976; Entrikin et al, 1977; Bhargava et al, 1977; Barnard & Barnard, 1979). AChE is a particularly useful enzyme for monitoring the success of chemotherapy in the dystrophic chicken because elevations of the enzyme occur in both plasma and muscle. Plasma levels can be studied to discover whether drug therapy suppresses the release of AChE from muscle. Both AChE and ψChE levels can also be assayed in dystrophic muscle to determine if the overproduction of enzyme, as well as AChE release, can be reduced. In addition, an investigation of the forms of AChE produced by muscle might indicate if therapy simply slows down total synthesis or release of enzyme, or if it actually restores normal synthesis of the AChE forms.

Plasma AChE only becomes significantly increased over normal levels after 3 weeks ex ovo. Therefore, any attempt to assay for chemotherapeutic affects before this age would be fruitless. However, since drug therapy as employed in this laboratory was usually started on d3 ex ovo, it was desirable to assay AChE levels as early as possible, in case particular drug dosages did not maintain an effect over a prolonged period. Hence, plasma was usually assayed between d35 and d45, when control levels of AChE are significantly elevated. In certain cases, where there was a substantial decrease in plasma levels, AChE was also assayed (usually between d44 and d64) in biopsy samples from the pectoral muscle of treated and untreated control dystrophic birds.

The drugs employed in this study are grouped into three categories. First, several anti-serotonin drugs were employed.

Bhargava et al (1977) detected slightly elevated levels of serotonin in plasma from dystrophic chickens. They proposed that serotonin is either indirectly damaging to dystrophic muscle by causing vasoconstriction and reducing blood flood or is directly harmful by interacting with the muscle membrane. They used two structurally unrelated serotonin antagonists, Cyproheptadine and Methysergide, to treat dystrophic chickens. They found reduced levels of several muscle enzymes in the plasma, as well as an improved flip number. Bhargava et al suggested that these drugs act either by improving the microvascular blood supply or by removing serotonin which might be exerting a direct, deleterious effect on the muscle fibre membrane.

A second category of drug is represented by diphenylhydantoin (DPH) which is an anti-myotonia drug. This drug was used, in combination with exercise, by Entriken et al (1977) to treat dystrophic chickens. They found that AChE levels in the PLD, as well as flip numbers, were virtually normal after 27-41 days of treatment. They suggested that the effect of this drug might be due to a stabilization of the muscle membrane since DPH has been shown to cause an alteration of membrane fluidity in erythrocytes from myotonic patients (Roses et al, 1975) and a stabilizing effect on neuronal membranes (Julien & Halpern, 1970). DPH alsoreduces the production and release of AChE from muscle cells cultured in vitro (Cisson et al, 1978). The light form of AChE (as detected by gel electrophoresis) is specifically diminished. This effect, however, is only selective for AChE at the lowest dosage employed, as higher levels have a generally toxic effect on the cells. It is, thus, unclear exactly what the response of muscle cells in vitro to the lowest dosage of DPH represents - it could be an adverse response of the cells to this chemical. In vivo, however, DPH does appear to cause an improvement in the functional, as well as biochemical, symptoms of dystrophy.

The third type of drug, N-isopropylamino-2-pyrimidine phosphate (IAPP), is neurotrophic. It has been used in the treatment of patients with leprous neuritis and dramatically improves their electromyographic patterns after four months of therapy (Sebille & Hugelin, 1980). Sebille & Hugelin did not suggest any cellular mode of action for this drug but stated that it has been shown to increase the outgrowth of neurites in vitro and in vivo.

7.2 Results and Discussion

A factor to consider in the interpretation of drug therapy results is the effect of drugs on body weight. Bhargava et al (1977) found that a general depression of body weight (ca 20%) occurred with three drug therapy regimes in the dystrophic chicken. They reported that no correlation was apparent between individual body weights and improvement of the dystrophic symptoms (e.g. flip number or plasma CPK level). However, Cisson et al (1978) have illustrated how too high a drug dosage can be quite toxic to cells in vitro. Toxicity in vivo is often reflected in a reduced gain in body weight. Preliminary results from other researchers in this laboratory indicate that a depressed body weight per se, without drug therapy, may cause lowered plasma enzyme levels. Therefore, drug dosages, as employed in this study, were chosen so as not to substantially alter weight gain. In evaluating the drug therapies which most effectively reduce AChE, note has also been taken of the weight gain. This is expressed as the percentage change in the mean of body weights of treated birds as compared to untreated control groups of dystrophic birds which were reared concurrently.

7.2.1. Anti-serotonin drugs

7.2.1.1 Sanomigran

Sanomigran (Pizotifen hydrogen malate) reduces plasma AChE approximately 35% from control dystrophic levels when given at

20-80mg/kg/day (Table 19). The most significant effect is produced with a dosage of 80mg/kg, but this level of drug also produces the largestdepression of body weight seen with this drug. Because Sanomigran may be slightly toxic, it was not employed at higher dosages. When employed at two moderate levels (25→40 and 40mg/kg) this drug does not alter the levels of AChE in the pectoral muscle (Table 20).

The percentages of AChE activity present in the L_2 and H_2 forms, as determined from gradients representing muscle samples from several different birds, are given in Table 21. The level of L_2 in the treated group (39%) is less than that in the control dystrophic group (64%). This might be considered to indicate a decreased production of L_2 AChE. However, since total AChE levels in the muscle are not significantly lowered, the reduced amount of L_2 does not truly represent decreased (or more normal) enzyme synthesis. Levels of L_2 in other groups of dystrophic birds between d32-60 are similarly 40-50% (see Table 6; 4.2). Therefore, the apparent difference in the percentage of L_2 in the Sanomigran treated birds may be due to random sampling error since so few chickens were tested -- higher than usual levels in the controls and lower than usual levels in the treated birds. Therefore, Sanomigran suppresses the release of AChE from dystrophic muscle but apparently does not reduce its excess synthesis in the muscle.

ψChE, however, is reduced in the pectoral muscle at both dosages; the effect is greater at the higher dosage. This result might be explained if ψChE is derived from the connective tissue or fat in dystrophic muscle. If drug therapy retards muscle degeneration, in general, then it could lower ψChE by reducing the amount of such tissues, even though AChE synthesis in muscle fibres may not be altered.

TABLE 19

THE EFFECT OF CHEMOTHERAPY ON PLASMA LEVELS

OF AChE IN THE DYSTROPHIC CHICKEN

Drug	Dosage (mg/kg)	Age (d)	n	% change in AChE	p[a]	Weight[b] change (%)
IAPP						
	20	47	5	-40	N.S.	
	30→40[c]	47	6	-73	<0.01	-20
	40	44	9	-3	N.S.	
	70[d]	35	6	-40	<0.05	-8
	165[d]	44	10	-23	N.S.	
	200-A[e]	45	9	-29	N.S.	
	"	85	9	-46	<0.002	+4
	200-B	35	10	-9	N.S.	
	300-A	45	8	-43	<0.05	-13
	300-B[f]	45	10	-27	N.S.	
	"	85	10	-45	<0.05	-5
	oral doses[g]					
	450	28	9	-12	N.S.	
	600	28	8	-21	N.S.	
	1000	35	10	-47	<0.01	-7
DPH						
	25	36	17	-6	N.S.	
	"	57	18	-5	N.S.	
	40	33	10	-41	<0.05	-5
DPH/Cyproheptadine						
	10/10 →	36	12	-50	<0.001	-28
	5/5[h]	58	10	-40	<0.01	-16
Cinanserin						
	40A	35	12	-44	<0.02	-15
	40B	35	8	-29	N.S.	-8
	60	35	10	-32	<0.05	-19
Sanomigran						
	20	50	10	-33	<0.05	-15
	25→40[i]	34	9	-34	<0.10	-9
	"	91	7	-34	<0.10	-19
	40	35	9	-37	<0.05	-9
	80	35	8	-35	<0.02	-21

TABLE 19 -- Continued

Drug	Dosage (mg/kg)	Age (d)	n	% change in AChE	P[a]	Weight[b] change (%)
Methiothepin						
	10	44	9	-5	N.S.	
	15	37	10	-56	<0.02	-11
	20	28	10	-45	<0.05	-10
	"	45	10	-32	N.S.	
	"	56	10	-33	N.S.	
Prajmaline						
	5	50	9	-34	<0.02	+1
Prajmaline/ Cyproheptadine						
	5/10	34	7	-39	N.S.	
Hepzidine						
	10	29	10	-5	N.S.	-5
	"	43	10	-14	N.S	-7
	"	58	8	-24	N.S.	-6

[a] P represents the level of significance as determined by the student T test. N.S. = not significant.

[b] The % change in mean body weight, as compared to the mean weight of a group of untreated dystrophic controls.

Drugs were administered as daily intraperitoneal injections, at a constant dosage from d3 ex ovo, except where the dosage was:

[c] increased on d34

[d] 3 X/day @ 55mg/kg/injection

[e] terminated on d36

[f] 3 X/week @ 300mg/kg/injection, terminated on d36

[g] given daily, oral doses

[h] decreased as indicated, on d31

[i] increased, as indicated, on d38

TABLE 20

THE EFFECT OF CHEMOTHERAPY ON AChE AND ψChE IN THE PECTORAL MUSCLE OF DYSTROPHIC CHICKENS

	Dosage (mg/kg/d)	Age (d)	n
IAPP			
	160	61	3
	control	61	3
	200	85	3
	control	85	3
oral dosage:			
	600	34	2
	control	34	2
	1000	44	3
	control	44	3
DPH			
	25	44	4
DPH/Cyproheptadine			
	10/10 → 5/5[a]	44	4
	control	44	4
Methiothepin			
	20	64	3
Sanomigran			
	25 →40[b]	43	2
	control	43	2
	40	63	3
	control	63	3

Drugs were generally administered by daily intraperitoneal injection from d3 ex ovo, except for the trials of IAPP where drug was administered orally, as explained in Table 19.

TABLE 20 -- Continued

AChE (U/g)	% change from control	ψChE (U/g)	% change from control
3.09 ± 0.88 3.14 ± 0.55	-5	0.702 ±0.138 0.707 ±0.134	-1%
2.44 ±0.59 2.59 ±0.56	-6	- -	
2.34 ±0.83 3.22 ±0.13	-27	1.01 ±0.13 1.63 ±0.06	-38
2.25 ±0.85 2.19 ±0.33	+3	1.36 ±0.07 1.89 ±0.31	-28
2.89 ±1.25	-6	0.966±0.152	+47
2.43 ±0.39 3.09 ±0.58	-21	0.633 ±0.060 0.656 ±0.079	-4
4.13 ±0.42	+27	0.176 ±0.027	-69
1.70 ±0.35 1.96 ±0.87	-13	0.598 ±0.139 0.679 ±0.162	-12
3.65 ±1.31 3.14 ±0.55	+13	0.555 ±0.221 0.707 ±0.134	-21

[a] The dosage was changed from 10/10 to 5/5 mg/kg on d31

[b] The dosage was changed from 25 to 40 mg/kg on d38

TABLE 21

THE EFFECT OF CHEMOTHERAPY ON THE REGULATION OF AChE FORMS IN DYSTROPHIC PECTORAL MUSCLE

Drug	Dosage (mg/kg)	n	Age (d)	L_2 (% of activity)	H_2 (% of activity)
Sanomigran	40	3	63	39	34
	control	3	63	64	18.5
IAPP	600 oral	2	34	56	24
	control	2	34	64	18.5

The terms L_2 and H_2 are defined in Table 4.

7.2.1.2 Cinanserin

Several different trials showed that Cinanserin reduces plasma levels of AChE by 29-41%, with varying degrees of significance. Because weight gain is affected somewhat at the highest dosage (60mg/kg), higher levels of this drug were not employed. Cinanserin is generally effective at the range of 40-60mg/kg in lowering AChE release into the plasma.

7.2.1.3 Methiothepin

Methiothepin reduces plasma AChE by 32-56% when employed at 15-20mg/kg and weight gain is not substantially altered. It is not effective at a lower level of 10mg/kg. AChE levels in muscle are not affected at 20mg/kg, while ψChE is reduced to almost normal levels. The reason for the dissimilar effect of Methiothepin on AChE and ψChE could be the same as that proposed for Sanomigran: connective tissue or fat may account for a substantial portion of ψChE (but not AChE) activity; such tissue may be reduced in amount in treated dystrophic muscle if therapy delays or prevents degenerative changes in muscle fibres.

7.2.1.4 Hepzidine

At the dosage tested (10mg/kg) Hepzidine only lowers AChE in the plasma 14% and is, thus, not an effective chemotherapeutic agent.

7.2.2 Anti-myotonia drugs

7.2.2.1 Prajmaline

Prajmaline, an anti-myotonia drug, significantly lowers plasma AChE levels by 34% when administered at 5mg/kg. It was also given in combination with Cyproheptadine, an anti-serotonin drug which was previously shown to be effective in reducing the levels of other enzymes in plasma of dystrophic chickens. The combined effect of these two drugs is a slightly greater reduction of plasma AChE which, however, is not statistically significant. Therefore, Cyproheptadine does not enhance the effect of Prajmaline as it has been shown to do with Methysergide (Bhargava et al, 1977).

7.2.2.2 DPH

DPH has previously been shown to be effective in reducing the symptoms of dystrophy in the chicken when employed at a dosage of 20mg/kg, 2 times a day (or 40mg/kg/day). In this study a dosage of 25mg/kg is shown to be ineffective in changing plasma or muscle levels of AChE. At a single daily dosage of 40mg/kg, however, plasma AChE levels are reduced 41%. When combined at a lower level (10mg/kg) with Cyproheptadine (at 5mg/kg) there is a highly significant 40-50% decrease in plasma AChE at both d36 and d58. The levels of AChE are also reduced by 21% in the pectoral muscle with this therapy regime. Although early weight gain (e.g. on d36) was affected slightly more by DPH than by other drugs, weight increased later when the drug dosage was reduced. AChE levels were still depressed on d58, even though the mean weight of the treated group had increased. Thus, the drug, and not solely a reduced body weight, appears to have been the real cause of the decrease in enzyme levels. Combining Cyproheptadine with DPH is, therefore, a successful combination therapy.

The previous therapy trial of DPH was combined with a program of exercise (Entrikin et al, 1977). These authors did not report any data on plasma enzyme levels, although reporting that the levels of AChE in the PLD muscle of treated birds were virtually normal between d27-41. It is unclear from their report if exercise enhances the effect of DPH in lowering AChE; by itself, exercise did not improve AChE levels or localization. No such exercise was employed in the present study. Also, since the PLD is less severely affected by dystrophy than the pectoral muscle, no direct comparison can be made between this and the previous work. However, DPH, as reported here, is apparently effective in reducing plasma levels of AChE. This could be due to a stabilization of the muscle membrane, as implied by Entriken et al.

DPH has been administered to dystrophic mice over several weeks (Silverman et al, 1978). These researchers found that DPH did not alter the abnormal internal Na:K ratio which occurs in the muscles of dystrophic mice, but it did cause an improvement in muscle function and in the electromyographic pattern. They concluded that the effect of DPH was not directed towards an alteration of the dystrophic determinant which causes the ion imbalance in the muscle. Instead, they believe that this drug acts on the membrane of the motoneurone. Since the dystrophic chicken and mouse are two quite different animal models for this disease, no direct comparison of the effect of DPH in these two systems can be made. However, the results of Silverman et al are similar to those reported here in that DPH does not appear to turn off the dystrophic abnormality in the muscle fibre; but this drug may be able to improve the general health of the dystrophic fibre, perhaps by stabilizing membranes.

7.2.3 A neurotropic drug

The effect of IAPP was examined over a wide range of doses. Results from different trials are somewhat variable, but this drug generally reduces plasma AChE by 20-45% when injected intraperitoneally. The most effective therapy is achieved with a dosage of 200mg/kg/day, a greater effect appearing later (on d85) than earlier (on d45). This is proof that IAPP substantially reduces AChE release over a considerable period of time - even at ages when AChE levels in untreated controls are greatly increased (i.e. d40-90). No substantial reduction in weight gain results from treatment with this drug, either, which indicates that it is relatively non-toxic.

A series of oral dosages was also investigated. Results show that the effectiveness of IAPP in reducing plasma AChE is directly related to the level of drug. The highest dosage (1g/kg) is most effective in significantly reducing plasma AChE (-45%). Within the pectoral

muscle, however, AChE is only slightly decreased at dosages of 160 or 200mg/kg or oral dosages of 600 or 1,000mg/kg; ψChE, however, is decreased. The forms of AChE in the pectoral muscle are also unaffected by IAPP therapy; L_2 remains predominant (Table 21). Since it is relatively non-toxic, however, higher dosages of this drug could be employed which might produce more dramatic effects; the amount of drug required for such studies was unavailable. The effect of IAPP on dystrophic muscle may be to stabilize the fibre membrane (or, possibly, the nerve membrane), as suggested by Sebille & Hugelin (1980) to explain its effect in nerve regeneration. However, at the dosages examined, it does not appear to be able to reduce AChE synthesis inside the muscle, although reducing the production of ψChE and release of AChE.

7.3 Summary

Several parameters, in addition to AChE, have been employed by other researchers in this laboratory to assess the effectiveness of the drugs discussed here in the therapy of dystrophy in the chicken. These include: the levels of other muscle enzymes in plasma (CPK and PK), physical tests (flip numbers and wing apposition) and histological examination of muscle. The results indicate that all three enzymes are usually affected to a similar extent by different drug therapies (Barnard & Barnard, 1979; Barnard et al, 1980a). Improvement in histopathology and functional tests also correlate well with the degree of enzyme depression (Barnard et al, 1980b).

Although Cisson et al (1978) argued that one particular drug, DPH, has a specific effect of decreasing L_2 AChE synthesis in tissue cultured cells, the effect of this and of several other chemotherapeutic drugs employed in this study in vivo appears to be a general depression of the release of muscle enzymes, but not a reduction of AChE overproduction.

All three categories of drugs employed reduce, to varying degrees, the release of AChE from muscle into the plasma. DPH, an anti-myotonia drug, was not found to be as dramatically effective as reported earlier by Entrikin et al, although its effect appears to be enhanced by using it in combination with Cyproheptadine. The most effective drug is IAPP, a neurotropic drug, which reduces plasma AChE 45% and is not toxic. Several anti-serotonin agents are also effective, but some have the disadvantage of being more toxic at higher levels (e.g. Cinanserin and Sanomigran). They may, therefore, be limited in their usefulness.

None of the drug therapies examined restore normal synthesis of AChE in the dystrophic pectoral muscle. However, the more normal histology of the muscle and its better functional performance indicate that a general improvement in muscle health was produced by some of the chemotherapeutic treatments. A common factor among the different types of drugs employed is that each group has been suggested as possibly acting by stabilizing membranes. It has been proposed (see 1.4.2.1) that the electrophysiological problems in dystrophic muscle may result from a problem in calcium conductance in the membrane. Flip numbers might be improved if this fault were corrected by stabilizing membranes during chemotherapy. AChE regulation is also very sensitive to events at the membrane (e.g. denervation, innervation), yet increased synthesis of this enzyme in muscle fibres does not appear to be greatly reduced with drug treatments. Thus, therapy may not actually be able to suppress the action of the dystrophic gene, as expressed in the abnormal synthesis of AChE. But, if the effect of chemotherapy is to stabilize the fibre membranes, it may, thus, decrease the overall leakage of muscle enzymes into the plasma and cause a general improvement in surface membrane integrity and muscle fibre morphology.

"It is not only fine feathers that make fine birds."

Aesop
"The Jay and the Peacock"

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