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Muscle Activity Pattern Regulates Postnatal Development of Acetylcholinesterase Molecular Forms in Normal Mice and Mice with Motor Endplate Disease

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We have studied the relative contributions of muscle activity and nerve-supplied materials to the regulation of AChE molecular forms during postnatal development of muscles in normal mice and in mice with motor endplate disease (*med* mice). Onset of this hereditary disease causes a progressive failure of evoked release of ACh from the motor neuron, which prevents contraction in muscles such as biceps and soleus. In these innervated but inactive muscles, one can examine the consequences of inactivity on the distribution of AChE forms. In normal mouse biceps the distribution of AChE forms, as shown by sucrose-gradient analysis, change substantially after birth; the most dramatic alteration is an increase in G_4 AChE from 15 to 45% of total AChE during the third postnatal week. AChE profiles in normal or *med* biceps are indistinguishable until 10–12 d after birth, but the change in distribution of AChE forms does not occur in *med* biceps nor in normal biceps denervated 2 weeks after birth. In contrast, the distributions of AChE forms in a predominantly slow muscle, the soleus, are similar in *med* and normal mice both early (10 d) and late (20 d) in the course of the disease, and the distributions are affected little by denervation. The profiles of AChE forms seen in normal soleus at all times studied resembled those seen in newborn biceps or biceps inactivated by denervation or the *med* disease. We conclude that neither innervation, age-dependent changes intrinsic to muscle, nor muscle activity is sufficient to induce the changes we see in AChE forms in biceps. These results support the hypothesis that neonatal, inactive, or tonically active muscles produce an intrinsic pattern of AChE molecular forms, and that a phasic pattern of activity induces a postnatal redistribution of the AChE molecular forms expressed by the muscle.

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Innervation regulates the production and properties of several macromolecules of the neuromuscular junction, including acetylcholine receptors (AChR) and AChE (reviewed in Dennis, 1981). Regulation of muscle properties by the nerve has been ascribed both to the influence of nerve-supplied "trophic" factors (Guth, 1968; Harris, 1974; Drachman et al., 1982) and to nerve-induced muscle activity, i.e., excitation and/or contraction, and its sequelae (Harris, 1981). Because muscle AChE is profoundly influenced by innervation, it has served as a paradigm for understanding the ways in which the nerve can influence muscle properties (e.g., Massoulié and Bon, 1982).

AChE exists in several distinct molecular forms in vertebrate skeletal muscles (Massoulié and Bon, 1982). There are at least 3 different globular forms, called G_1, G_2, and G_4 (the subscript designates the number of catalytic subunits), and at least 3 tailed, asymmetric forms, called A_4, A_8, and A_{12} (Bon et al., 1979). The A_{12} form, which is probably responsible for hydrolysis of ACh released from the nerve (McMahan et al., 1978), has been of particular interest because it is preferentially localized to synaptic regions of muscle (Hall, 1973; Vigny et al., 1976; Younkin et al., 1982) and because it is selectively lost after denervation of rat and chicken muscles (Hall, 1973; Linkhart and Wilson, 1975; Vigny et al., 1976). This effect of innervation on A_{12} AChE is mediated in part by muscle activity, because activity is required for production of A_{12} AChE at denervated rat endplates *in vivo* (Weinberg and Hall, 1979) and in vertebrate muscle *in vitro* (Rieger et al., 1980; Rubin et al., 1980; Brockman et al., 1984; but see Lappin and Rubin, 1985). We therefore wondered how muscle activity affects production and maintenance of A_{12} and other forms of AChE *in vivo*, and to this end we sought a means to inactivate developing mammalian neuromuscular junctions *in situ*.

We have made use of mice with motor endplate disease (*med* mice), a heritable, recessive disorder that causes progressive muscle weakness and death by about 21 d after birth (Duchen, 1970). This disease indirectly inactivates muscles by blocking evoked, but not spontaneous, release of ACh from the neuron (Duchen and Stefani, 1971; Harris and Ward, 1974). One can therefore study the severely affected limb muscles (Duchen, 1970) from before the time of onset of symptoms at about 10 d after birth until the death of the animal, to see how development of the properties of innervated muscle is affected by inactivity.

We have extensively characterized the changes of AChE molecular forms in postnatal developing mouse muscles and have investigated the roles of muscle activity and innervation in inducing those changes. We find that *med* or surgical denervation causes a selective loss of G_4, but not of A_{12}, AChE in predom-

inantly fast muscles such as biceps brachii or diaphragm, while in the predominantly slow soleus muscle, *med* or denervation has little effect on the distribution of AChE forms. These results indicate that in developing muscle, it is the pattern of muscle activity, rather than activity per se, that regulates the distribution of AChE molecular forms. A similar conclusion has been reached by Lomo et al. (1985), on the basis of their electrical stimulation of denervated adult rat muscles. Our results confirm and extend earlier studies of AChE in developing hindlimb (Rieger et al., 1983) and forelimb (Reiness and Yeakley, 1983) muscles of mice with a less severe form of motor endplate disease (*med*^{J}).

Materials and Methods

Mice. The *med* mutation is carried in a stock of mice that also has a closely linked dominant mutation, Caracul d (*Ca*^{d}), that causes a curly coat and whiskers (Searle, 1970). Breeding pairs of *med* +/+ *Ca*^{d} mice were obtained from Dr. A. G. Searle, MRC Radiobiology Unit, Harwell, UK, and used to establish a breeding colony that is now maintained by us. Because the original stock was not inbred, having both strains C3H and 101 in its background, we have used a circular-pair breeding scheme (Kimura and Crow, 1963) to avoid inbreeding and maintain the vigor of the animals and the size of litters.

Med mice (*med* +/*med* +) can be identified by their straight whiskers at about 2 d after birth (Searle, 1970), well before the onset at 10–12 d of age of neurological symptoms that lead to death at about 21 d (Duchen, 1970). The mice that are heterozygous (*med* +/+ *Ca*^{d}) or homozygous wild type (+ *Ca*^{d}/+ *Ca*^{d}) at the *med* locus show no neurological symptoms and have curly coats; they cannot be distinguished until 3–4 weeks of age, when the wild-type mice, which are also homozygous for the Caracul mutation, show loss of hair. Therefore, unaffected neonatal littermates of *med* mice used in these studies were of both (unknown) genotypes (i.e., + *Ca*^{d}/? ?). We have treated them together as "normal" mice, because we saw no differences between the distribution of AChE forms in older animals in which we could distinguish the genotypes.

Chemicals. General chemicals were purchased from Sigma Chemical or J. T. Baker. ^{3}H-Acetylcholine iodide (^{3}H-ACh) with a specific activity of about 50 Ci/mmol was obtained from New England Nuclear.

Analysis of AChE molecular forms. The mice were killed by cervical dislocation and the biceps brachii (long head) was dissected and held on ice in Krebs–Ringer solution (4 mM KCl, 138 mM NaCl, 1 mM $MgSO_4$, 12 mM $NaHCO_3$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) or Hanks' Balanced Salt Solution with $NaHCO_3$ (HBSS) from Gibco. All subsequent procedures were conducted at 4°C.

Weighed muscles (3–20 mg) were homogenized in 0.4 ml of buffer containing 10 mM Tris-HCl, pH 7.4, 1 M NaCl, 20 mM EDTA, 5 mM EGTA, 1.0% Triton X-100, 0.1 μg/ml pepstatin, 1 μg/ml leupeptin, and 20 U/ml aprotinin. *N*-Ethyl maleimide (5 mM) was occasionally added, but because it had no discernible effect on the distribution of AChE forms on sucrose gradients and could interfere with AChE assays, it was usually omitted. Muscles were homogenized with a Brinkmann Polytron, and the suspensions centrifuged at 25,000 × *g* for 15 min.

Samples of the supernatants (0.2 ml) were loaded on 12.4 ml 5–20% sucrose gradients made in the homogenization buffer (without *N*-ethyl maleimide) and centrifuged for 22 hr at 40,000 rpm in a Beckman SW40 rotor. Approximately 0.25 ml (25-drop) fractions of the gradients were collected from the tube bottom and analyzed for AChE activity by the method of Johnson and Russell (1975), as modified by Younkin et al. (1982). Briefly, 0.05 ml of reagent solution containing 3×10^{-4} M ^{3}H-ACh (3×10^{5} dpm) in 0.1 M KPO_4, pH 7.0, was added to each fraction. The tubes were mixed and incubated at room temperature for 30–120 min, and then the reaction was stopped by acidification. Protonated ^{3}H-acetate was extracted by adding 4 ml of nonaqueous scintillant containing 10% isoamyl alcohol. Samples were counted on an LKB 1211 or a Beckman LS 100 scintillation counter. Sedimentation coefficients were determined using β-galactosidase (16 S) and alkaline phosphatase (6.1 S) as standards. In the portion of the supernatant that was not loaded on the gradients, total AChE activity was determined as described above, and total protein was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard. AChE specific activity was expressed as substrate (pmol) hydrolyzed/min/mg protein to allow direct comparisons among gradients. Enzymatic activity in the muscles studied was at least 90% AChE, as determined by use of the inhibitor of nonspecific cholinesterase, *iso*-OMPA (tetraisopropyl pyrophosphoramide) (Sigma).

We estimated the proportion of each of the AChE forms graphically from plots of AChE activity versus fraction number, such as are shown in Figure 2. The G_1 and G_2 forms were not well resolved on these gradients, and they were therefore considered together.

For surgery, mice were anesthetized with pentobarbital, and the biceps brachii was denervated by cutting the musculocutaneous nerve at its point of departure from the brachial nerves. The soleus was denervated by cutting the sciatic nerve at mid-thigh. AChE forms in the muscles were analyzed 5–7 d after denervation.

To inactivate AChE *in vivo,* mice were kept anesthetized with pentobarbital, the surface of the biceps brachii was exposed, and the muscle was treated first with 2 μM α-bungarotoxin for 3 hr, then with 1 mM diisopropyl fluorophosphate (DFP) or vehicle (0.1% propylene glycol) for 3 hr (Salpeter et al., 1979). The muscle was rinsed with HBSS, the skin was clipped closed, and the mouse was allowed to recover for various lengths of time before AChE analysis.

Results

Total AChE in neonatal med *and normal biceps*

We first examined the changes in total AChE content of mouse biceps brachii muscle (long head) in normal mice and *med* littermates in the first 4 weeks after birth. The total AChE in normal mouse biceps increased 3–4-fold between postnatal days 7 and 24 (Fig. 1*a*). In contrast, AChE content in the biceps of *med* mice, while initially indistinguishable from that of the unaffected animals, did not increase during the same period.

The protein content of the muscles changed in a similar way to that of AChE content. In the normal mice, the muscle grew considerably during this period, with the total protein again increasing 3–4-fold (Fig. 1*b*). In contrast, the *med* biceps atrophied after about 14 d, declining to about half its maximum protein content by the time of the animal's death. Thus, the specific activity of AChE in both unaffected and *med* mice remained relatively constant during this postnatal period (Fig. 1*c*), although the specific activity was always about twice as high in unaffected mice.

Distribution of AChE forms in developing biceps

We next studied the distribution of AChE molecular forms in developing biceps muscles by separating the forms on sucrose velocity sedimentation gradients. In both normal and *med* mouse biceps we identified 5 distinct molecular species that had approximate sedimentation coefficients of 16, 13, 10, 6.5, and 4 S (Fig. 2), corresponding to the A_{12}, A_8, G_4, G_2, and G_1 forms described by Bon et al. (1979). If there was any 9 S (A_4) AChE in these muscles, it was not well resolved on our gradients.

At 7 d after birth, the abundance and relative distribution of the various forms were similar in normal and *med* mouse biceps (Fig. 2*a*), with A_{12} and G_1 the most prominent forms. By 16 d after birth, the distributions of AChE forms in normal and *med* biceps were distinct (Fig. 2*b*). In the unaffected mice, the A_{12} and G_1 forms were still prominent, but G_4 had become the second most abundant form. The *med* biceps showed a decrease in A_{12} AChE, no increase in G_4, and a substantial decline in the G_1 form compared to the profile at 7 d after birth. These trends continued, so that by 20 d after birth, G_4 AChE was the most abundant form in normal biceps, A_{12} was also prominent, and G_1 levels had declined (Fig. 2*c*). The *med* biceps had less of all forms than normal, but the A_{12} remained the most common form, G_1 had become a minor component, and no increase in the amount of the G_4 form was ever seen.

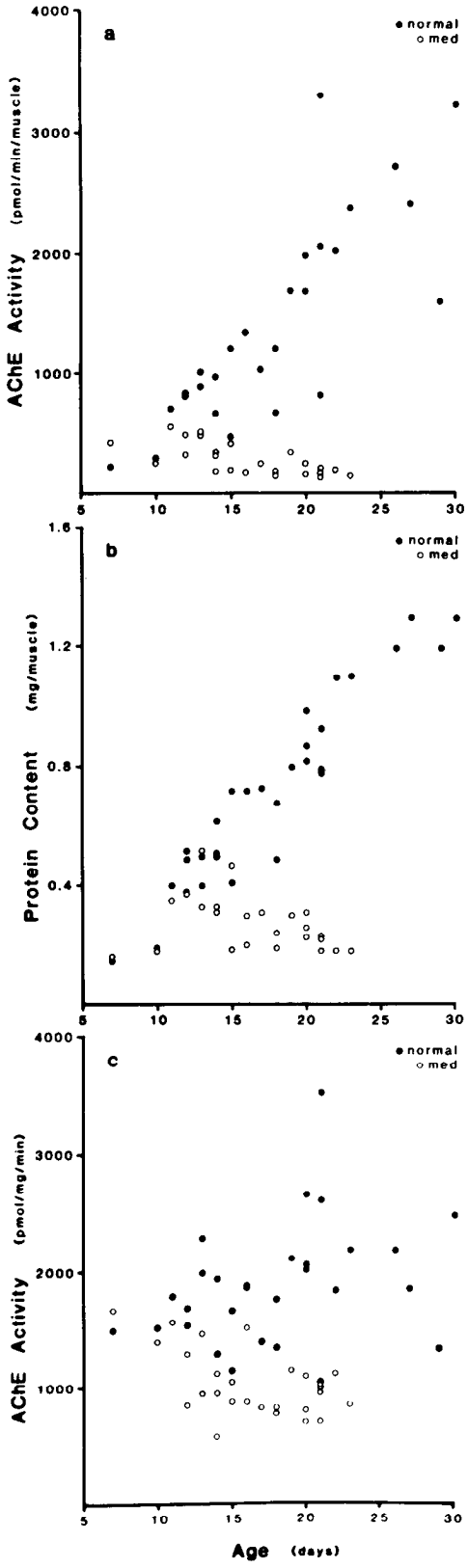

Figure 1. Postnatal changes in AChE activity and protein content in biceps brachii muscle of *med* and normal mice. Whole muscles from normal (●) or *med* (○) mice were analyzed at the age shown for (*a*) total AChE activity or (*b*) protein content, as described in Materials and Methods. (*c*) AChE specific activity, calculated from the data in *a* and *b*, is shown for normal and *med* biceps as a function of the age of the mouse.

A large number of sedimentation profiles such as those shown in Figure 2 were used to determine the proportion of each AChE form in normal and *med* biceps at various times after birth (Fig. 3). The proportion of A_{12} AChE in normal biceps declined slightly from about 35% to about 25% of the total (Fig. 3*a*), because the amount of G_4 increased, while the amount of A_{12} remained relatively constant (Figs. 2, 3*b*). In *med* biceps the fraction of A_{12} AChE stayed constant at around 40% of the total AChE (Fig. 3*a*), i.e., the declines in total and in A_{12} AChE were proportionate. In normal mice, G_4 increased dramatically from about 15% of total AChE at 12 d to over 40% in mature animals, while only a slight increase in the percentage of G_4 AChE from 15 to 20% of total AChE was seen in the *med* mice (Fig. 3*b*). The fraction of G_2 plus G_1 forms (which we considered together) declined from 50% of the total at 1 week to 25% by 4 weeks in normal biceps, mirroring the increase in G_4 AChE (Fig. 3*c*). These forms declined more rapidly to 20% of AChE in *med* mice (see Fig. 2). Finally, the A_8 form, which was only 5% of AChE in normal mouse biceps throughout development, increased in *med* biceps from 5 to 20% of AChE, becoming nearly as prominent as the G_4 or G_1 plus G_2 forms in these muscles (Fig. 3*d*).

Thus, not only does total AChE in unaffected biceps increase postnatally, but there is a dramatic increase in the abundance of the G_4 form relative to other molecular forms. Onset of the effects of the *med* mutation prevents these changes and reduces the relative proportion of the G_1 form as well. Both the normal process of development and the consequences of the *med* mutation therefore alter the distribution of AChE forms in this muscle.

Effect of denervation on AChE forms in neonatal biceps

These results suggested to us that muscle activity was necessary to produce the developmental changes in AChE forms in biceps and that the *med* mutation prevented these changes by blocking muscle activity. We tested this idea by denervating *med* and normal biceps. If AChE is regulated by activity, denervation should affect AChE in normal biceps, but not in already inactive *med* muscles. Denervation of normal biceps in 2-week-old mice caused a large decrease of total AChE, reflecting mostly declines in the G_4 and G_1 plus G_2 forms; A_{12} was the most abundant form remaining (Fig. 4, *a, b*). The effects of denervation and *med* on AChE in the biceps were thus similar. On the other hand, denervation of *med* biceps had only a minor effect on the content of all AChE forms except G_4, which was further reduced (Fig. 4, *c, d*). Since denervation of the *med* muscle should eliminate any remaining activity, as well as any nerve-derived trophic materials, these results indicate that the effects of *med* on AChE are mediated predominantly by muscle inactivity caused by the mutation.

Because A_{12} AChE is often selectively lost after denervation of vertebrate muscle (Massoulié and Bon, 1982), we wondered whether the persistence of the A_{12} form after denervation was unique to mouse biceps. We found that A_{12} was also the least affected form in diaphragm muscle denervated for 5 d (data not shown). We also confirmed that the A_{12} form was mainly located in synaptic regions of both biceps and diaphragm by dissecting the muscles into endplate-free and endplate-containing regions and analyzing these separately on sucrose gradients (cf. Gisiger and Stephens, 1983; Inestrosa et al., 1983; Rieger et al., 1984a). Thus the A_{12} form, though concentrated at endplates, is not

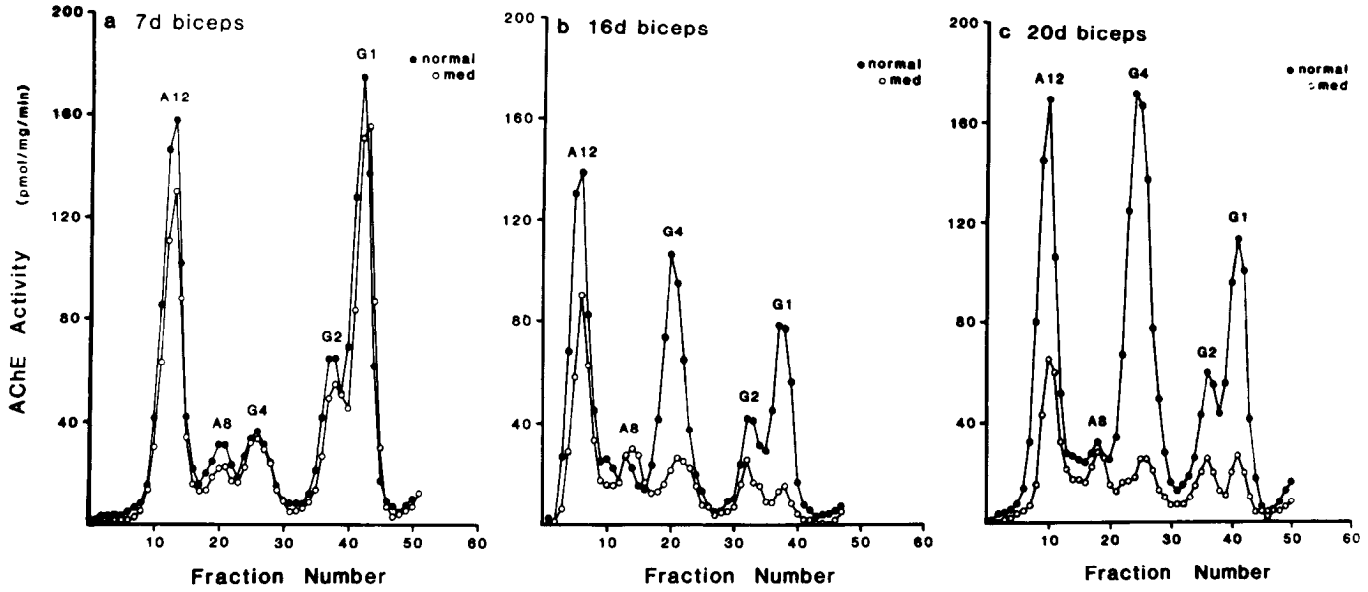

Figure 2. Distribution of AChE molecular forms in biceps brachii from mice of different ages. Muscles from normal (●) or *med* (○) mice at postnatal day 7 (*a*), 16 (*b*) or 20 (*c*) were analyzed for AChE molecular forms on sucrose velocity sedimentation gradients, as described in Materials and Methods. The bottom of the gradient is to the left (*Fraction 1*). The identification of the 5 distinguishable AChE forms is based on their sedimentation coefficients (Bon et al., 1979).

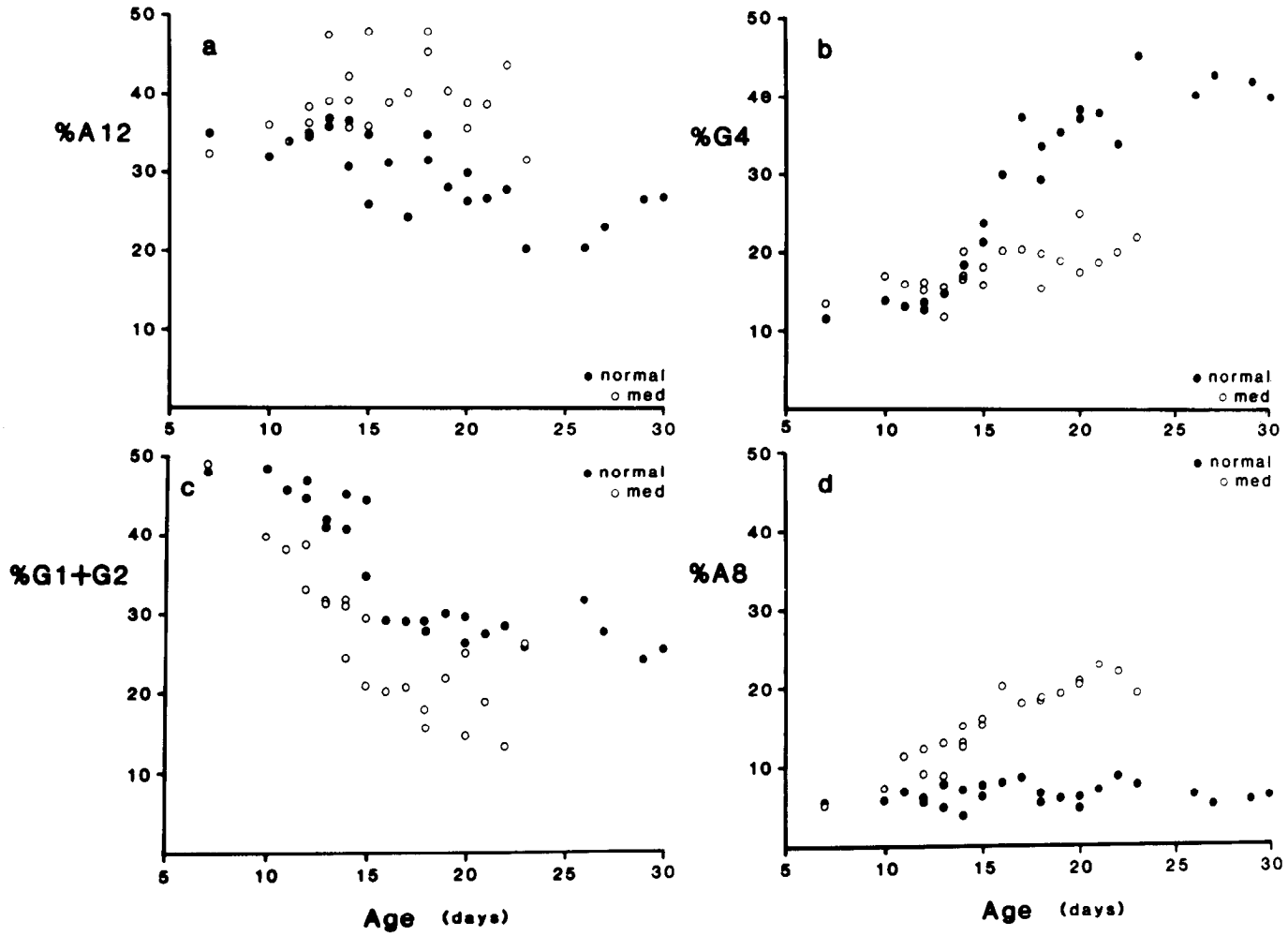

Figure 3. Changes in proportion of AChE molecular forms in biceps brachii of mice from 1 to 4 weeks after birth. The fraction of total AChE represented by each form was estimated graphically from data such as those shown in Figure 2. The percentage of total AChE of the (*a*) A_{12}, (*b*) G_4, (*c*) $G_1 + G_2$, and (*d*) A_8 forms is shown for normal (●) and *med* (○) biceps.

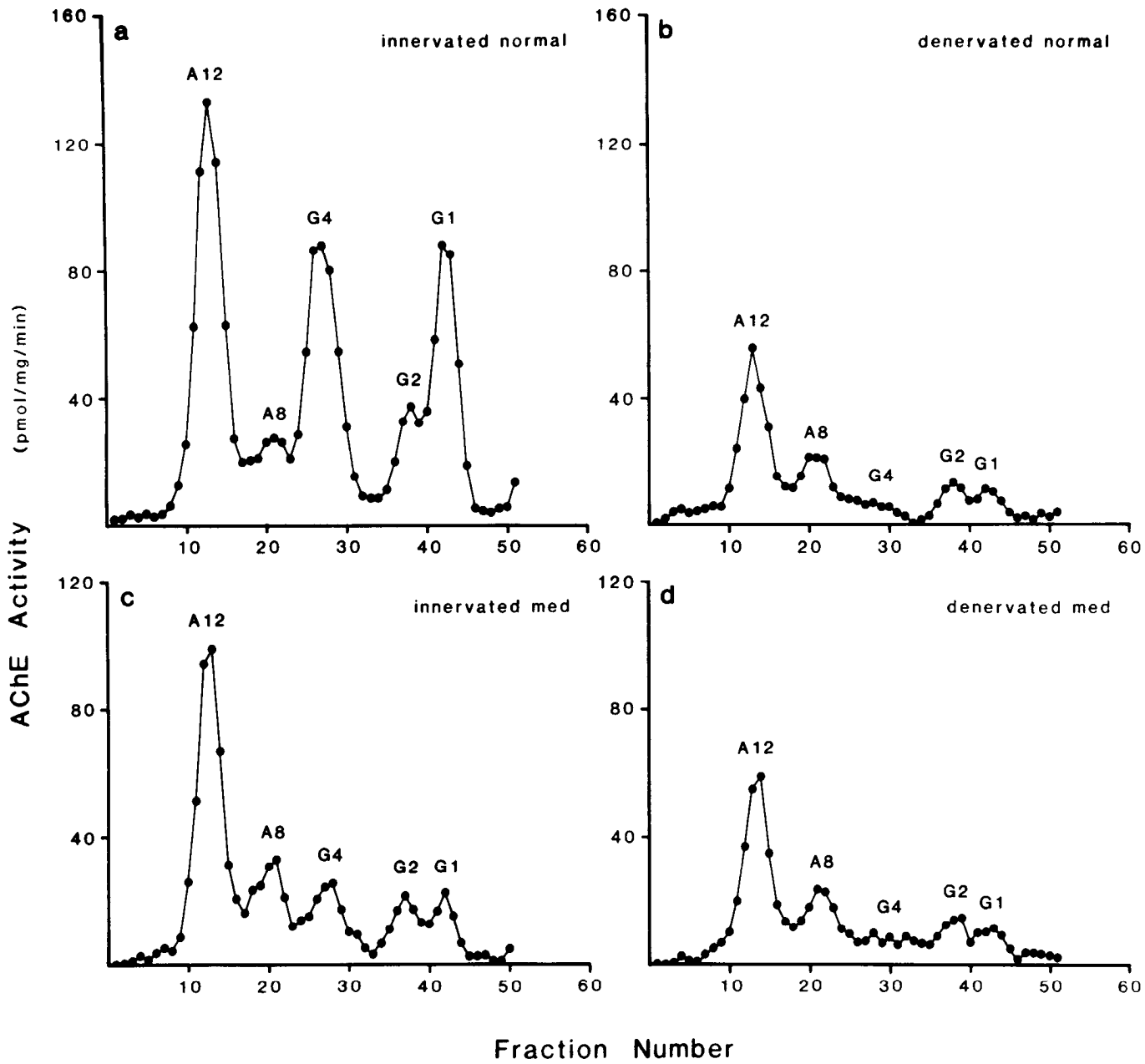

Figure 4. Effect of denervation on AChE forms in biceps brachii. The biceps of normal mice and *med* littermates were denervated 14 d after birth, then removed and analyzed for AChE molecular forms 7 d later, as described in Materials and Methods. Profiles for innervated and denervated biceps from a normal mouse are shown in *a* and *b*, respectively. Profiles for innervated and denervated biceps from a *med* mouse are shown in *c* and *d*. *b* and *d*, Location of the G_4 form estimated by analogy with its location on other gradients in which this form was more prominent. In all cases, the bottom of the gradient is *Fraction 1*.

preferentially lost following denervation or onset of *med* symptoms in these muscles.

AChE forms in soleus muscle

Because Lømo et al. (1985) have found that patterns of muscle activity may regulate the distribution of AChE in adult rat muscles, and because biceps and diaphragm are predominantly fast muscles, we also examined AChE forms during development in a predominantly slow muscle, the soleus. We found that there was little postnatal redistribution of AChE forms in this muscle; i.e., the distribution of forms was virtually identical in soleus muscles of 10 and 20-d-old normal mice (Fig. 5), except for a decrease in G_1 AChE. At all ages, the major form was A_{12}, and there were only minor amounts of the globular forms, especially G_4.

Surprisingly, we found little difference in the distribution of AChE specific activity between the *med* and normal soleus (Figs. 5; 6, *a, c*), although the *med* soleus was atrophied and had less total AChE. Denervation also had little effect on the distribution of AChE forms in either *med* or normal soleus (Fig. 6, *b, d*). Thus the distribution of forms in soleus, unlike that in biceps, is relatively insensitive to innervation or activity. The AChE profile in normal soleus muscle closely resembles the profile of immature biceps muscle or of biceps muscle that has been in-

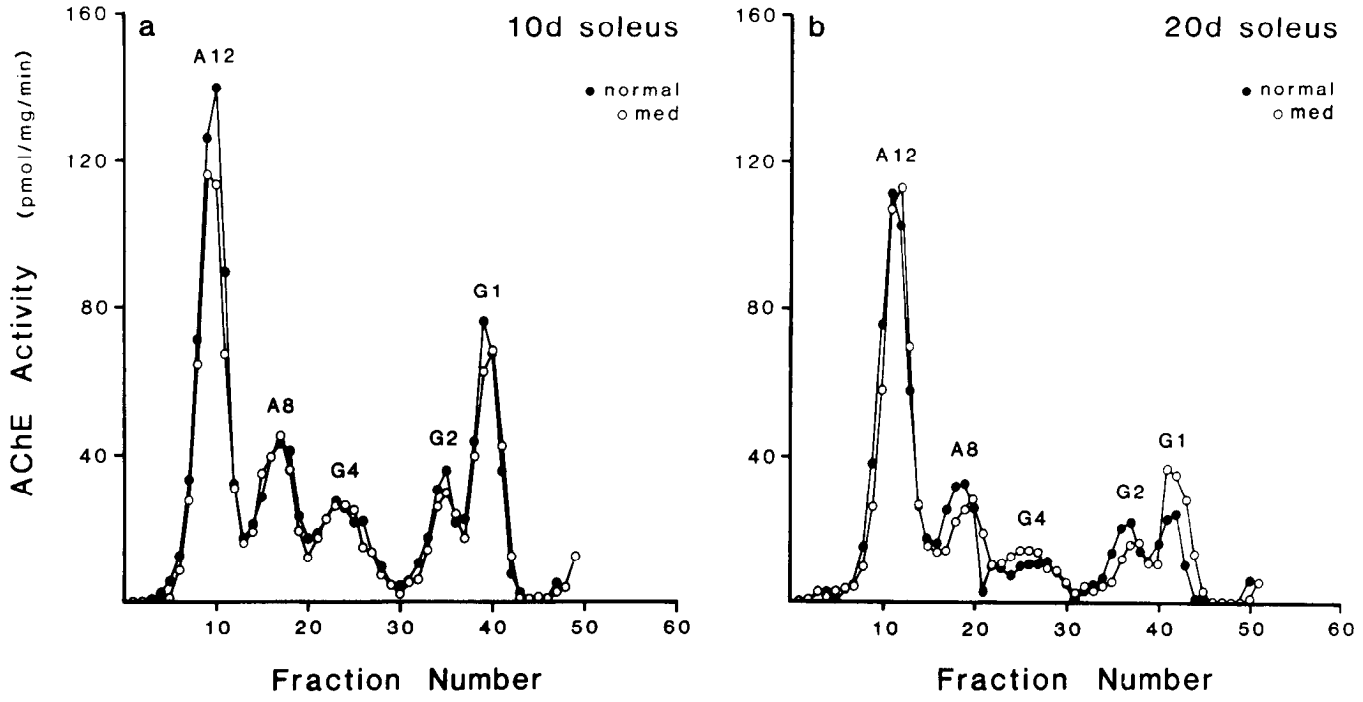

Figure 5. Distribution of AChE molecular forms in soleus from 10- and 20-d-old mice. Normal (●) and *med* (○) soleus muscles from littermates were assayed for AChE forms on sucrose velocity sedimentation gradients, as described in Materials and Methods. Shown are profiles from 10-d-old (*a*) and 20-d-old (*b*) mouse soleus.

activated by denervation or by the onset of motor endplate disease.

Biosynthesis of AChE in denervated and med *muscles*

Because the half-life of endplate AChE (mostly A_{12}) in denervated rat diaphragm is about 12 hr (Newman et al., 1984), it seemed likely that the A_{12} AChE present in denervated or *med* muscles was produced by continued synthesis. However, it is possible that this A_{12} might represent residual AChE molecules that existed before denervation or onset of the *med* symptoms. To distinguish between these possibilities, we used the irreversible AChE inhibitor, DFP, to block all AChE in denervated biceps, and then we followed the reappearance of newly synthesized AChE. As shown in Figure 7, denervated normal biceps was capable of synthesizing all forms of AChE, including A_{12}. We obtained similar results in 18 d *med* biceps, although we were unable to completely inactivate preexisting AChE, especially A_{12}, with DFP, probably because diffusion of DFP into these badly atrophied muscles was poor. Persistence of A_{12} AChE in denervated and, probably, *med* biceps is thus at least partly the result of its continued synthesis.

Discussion

Normal muscles

We have examined the distribution of AChE molecular forms in mouse biceps brachii and soleus muscles in the first 3 weeks following birth. We find that the profile of forms characteristic of adult muscle is present in the soleus shortly after birth; the biceps, in contrast, undergoes a profound alteration in the distribution of molecular forms before acquiring its mature AChE profile during the third postnatal week. From their study of various mouse muscles, Gisiger and Stephens (1983) have proposed that adult muscles can be grouped into different classes according to their characteristic AChE profiles; we find that the adult biceps has a profile similar to the one they observed for plantaris and extensor digitorum longus muscles (see also Skau and Brimijoin, 1981). These profiles are not invariant properties of the muscle, however; our results demonstrate that muscles can acquire these characteristic adult AChE profiles by a dynamic process that involves substantial postnatal redistribution of the AChE forms.

In biceps this redistribution is primarily of globular AChE forms, because the content of asymmetric A_{12} and A_8 forms changes little after birth (Figs. 2, 3; see also Rieger et al., 1984a). Rather, there is a large decrease in G_1 AChE and a dramatic increase in G_4 AChE during the third postnatal week (Figs. 2, 3). A similar finding has been reported for mouse gastrocnemius muscle (Rieger et al., 1983). This shift of forms may represent conversion of the G_1 to the G_4 form (Massoulié and Bon, 1982), but our data are not instructive on this point. Gisiger and Stephens (1983) found that the content of asymmetric AChE forms was relatively constant among several fast-twitch muscles in mouse, and that differences were primarily seen in the content of globular forms. We find a similar situation within a single muscle, biceps brachii, at different times. Thus, globular AChE content appears to be affected more than asymmetric AChE content by intrinsic or neuronal influences in mouse fast-twitch muscles.

The postnatal change in the profile of AChE forms in biceps clearly depends on the motor neuron, because denervation prevents it (Fig. 4). On the other hand, the distribution of AChE forms in soleus is not dependent on the motor neuron, because denervation has little effect on AChE in this muscle (Fig. 6). Thus, the effects of innervation on AChE are complex and differ

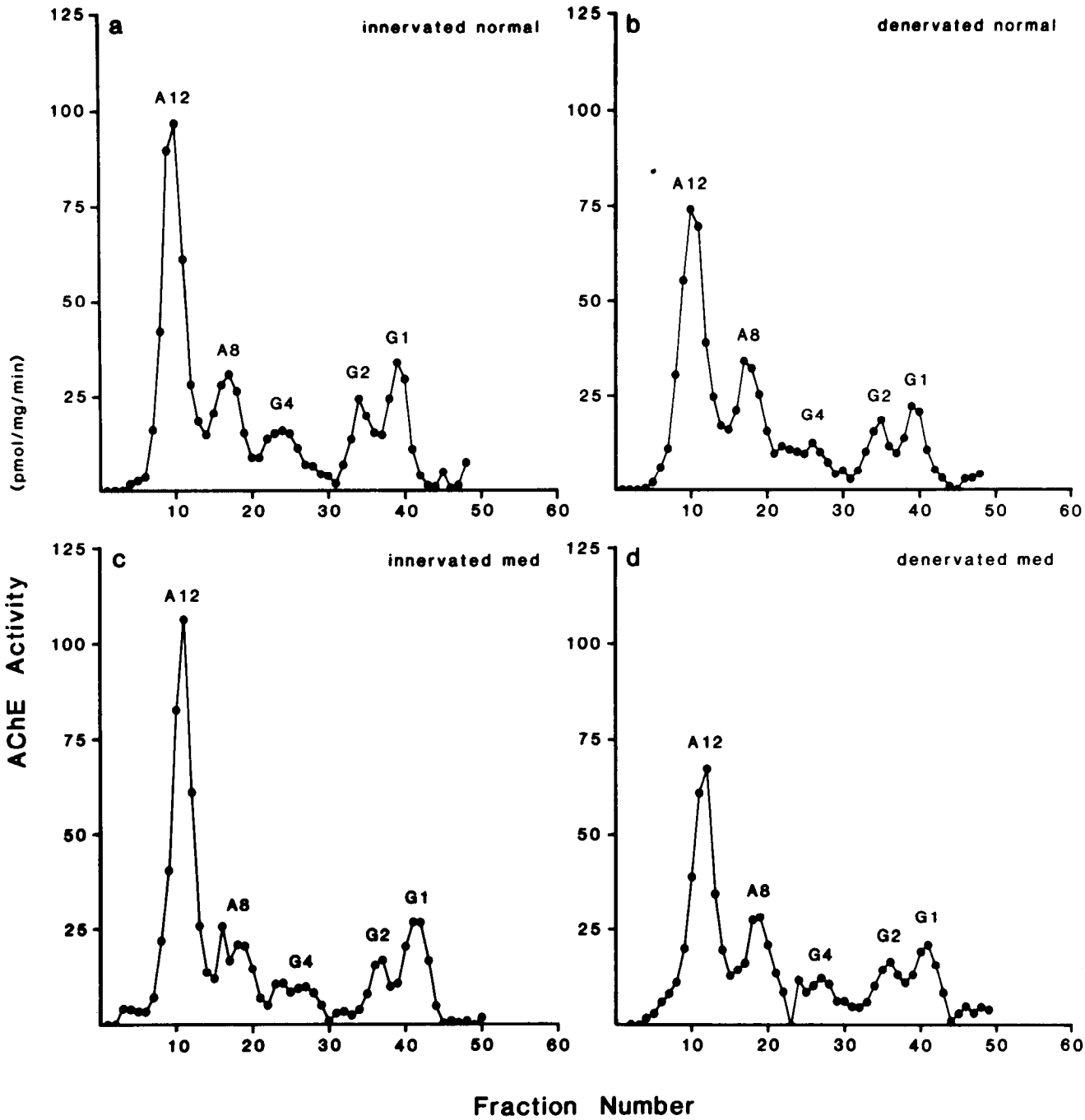

Figure 6. Effect of denervation on AChE forms in soleus. The soleus was denervated in 14-d-old normal and *med* mice, then removed and analyzed for AChE forms 7 d later. Shown are profiles from soleus muscles of *med* and normal littermates: (*a*) normal innervated; (*b*) normal denervated; (*c*) *med* innervated; and (*d*) *med* denervated.

among the various muscles in mice. In order to determine what role muscle activity plays in regulating AChE in biceps, we made use of mice with motor endplate disease.

Med *mice*

The use of *med* mice to assess the role of activity in muscle development presupposes that the lesion affects the muscle indirectly by acting on the motor neuron, and that production, transport, or release of putative trophic factors is not altered by the mutation. The nature of the *med* mutation is unknown, but the available morphological, physiological, and developmental studies indicate that *med* does affect the muscle indirectly, probably by interfering with the ability of motor neuron action potentials to be conducted in axons (Angaut-Petit et al., 1982) or to invade nerve terminals (Duchen and Stefani, 1971; Harris and Pollard, 1985). Stimulation of the nerve fails to evoke a response in the muscle, but the muscle contracts when directly stimulated (Duchen and Stefani, 1971; Harris and Ward, 1974). Further, in the presence of 4-aminopyridine, stimulation of the nerve sometimes induces endplate potentials or action poten-

tials in the muscle (Harris and Pollard, 1985). Thus, the muscle remains capable of responding to ACh from the neuron, and of excitation and contraction. Moreover, if muscles from *med* mice are minced and transplanted to normal hosts, they develop normally (Zacks and Sheff, 1977), again indicating that the muscle is spared direct effects of the lesion.

In contrast, the nerve fails to release transmitter in response to stimulation, as noted, and shows partial dysmyelination and an increase in the number of Na^+ channels by 9–10 d after birth (Rieger et al., 1984b). This is 2–3 d before onset of symptoms, and before detectable changes in AChE (this paper) and extrajunctional AChR levels in muscle (J. M. Yeakley and C. G. Reiness, unpublished observations). Several lines of evidence, therefore, implicate the nerve as the primary site of the lesion.

Although the muscle is inactive, the machinery for neuromuscular transmission remains intact in *med* mice. The nerve remains in close contact with muscle (although terminal sprouting is seen in some, but not all, muscles) (Duchen, 1970; Pincon-Raymond et al., 1983), and spontaneous release of ACh occurs at the normal rate (Duchen and Stefani, 1971; Harris and Ward, 1974). The postsynaptic junctional membrane appears to be normal ultrastructurally, with pronounced junctional folds (Duchen, 1970; Pincon-Raymond et al., 1983). The sensitivity of miniature endplate potential frequency to elevated K^+ or black widow spider venom is similar in normal and *med* mice, and ACh stores appear comparable in nerve terminals in both (Harris and Pollard, 1985). These results indicate that the enzymes necessary for synthesis and storage of ACh and the cellular components necessary for its release are present in *med* nerve terminals, and are therefore presumably moved by normal axonal transport or axoplasmic flow. The continuous spontaneous secretion of ACh at *med* neuromuscular junction suggests that other substances can be transported and secreted normally in these animals. While we cannot rigorously exclude the possibility that some material, dependent solely on evoked release, is absent or defective in affected neurons, we believe that the simplest explanation of these results is that the effects of the *med* mutation are caused by muscle inactivity. That is, the muscle remains innervated (i.e., the nerve remains juxtaposed), the nerve can spontaneously release ACh and possibly trophic factors, but the muscle becomes inactive because of defective conduction in the nerve. We have therefore exploited this system to determine how inactivity affects developmental changes of the AChE forms in muscle in the continued presence of the nerve.

The onset of symptoms of the *med* mutation at 10–12 d after birth prevents the changes in the profile of AChE forms that normally occur in the biceps. This is most obvious for G_4 AChE because *med* blocks the dramatic increase in this form that takes place in the third postnatal week. The G_1 and A_{12} AChE forms are also reduced by the disease, although not as extensively as G_4, and the A_8 form is increased. Muscle activity is therefore required to maintain the distribution of AChE forms in biceps and to induce the postnatal increase in G_4 AChE.

The soleus differs from biceps in its response to the *med* mutation, just as it differs in its response to denervation. Little effect of the *med* mutation is seen on the distribution of AChE forms in this muscle, so that the pattern in normal and *med* muscles is indistinguishable (Fig. 5). It is not that soleus is spared the effects of the disease, because we have confirmed that stimulation of the nerve does not elicit muscle contraction (not shown), and others have shown that there is extensive ultraterminal sprouting of the neurons in *med* soleus (Pincon-Raymond et al., 1983). Thus inactivity, like denervation, has little effect on the maintenance of the profile of AChE forms in soleus.

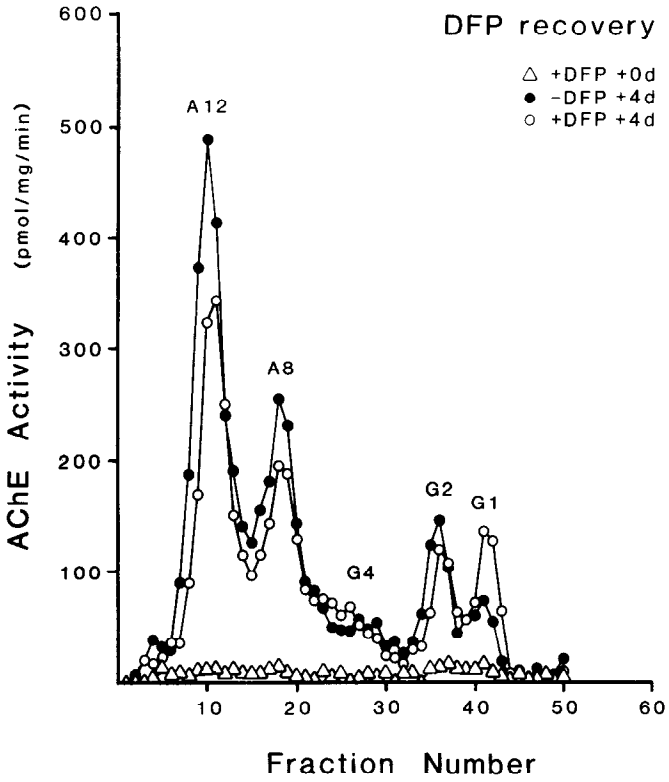

Figure 7. Recovery of AChE forms after DFP blockade. Biceps muscles were denervated in 14-d-old normal mice, and 4 d later AChE in the muscles was inactivated with DFP, as described in Materials and Methods. Treated muscles were analyzed for AChE forms immediately (△) or after 4 d recovery (○). AChE profile of the untreated contralateral muscle is also shown (●).

Regulation of AChE in mouse muscle

A_{12} AChE. In rat muscle, denervation causes a severe and selective loss of A_{12} AChE, the form that is predominantly concentrated in synaptic regions (Hall, 1973; Vigny et al., 1976; Younkin et al., 1982). Moreover, production of A_{12} AChE in cultured rat muscle requires muscle activity because TTX blocks its production and causes its loss (Rieger et al., 1980; Brockman et al., 1984). In chickens a similar loss is observed from denervated or inactivated muscles (Linkhart and Wilson, 1975; Rubin et al., 1980). It has therefore been suggested that A_{12} AChE is selectively regulated by innervation, but this conclusion seems inapplicable for mouse muscle. Although A_{12} AChE is concentrated in synaptic regions of mouse muscle (Gisiger and Stephens, 1983; Rieger et al., 1984a; and this work), substantial amounts of A_{12} AChE are found in denervated mouse muscles (Skau and Brimijoin, 1981; Rieger et al., 1983; and this work), in muscles inactivated by murine dystrophy (Skau and Brimijoin, 1981; Gisiger and Stephens, 1983) or motor endplate disease (Rieger et al., 1983; and this work), or in cultured mouse muscles inactivated by TTX (Inestrosa et al., 1983; Rubin et al., 1985). Thus, neither innervation nor muscle activity is necessary for synthesis of A_{12} AChE by mouse muscle, a finding that has also been reported for rabbit (Bacou et al., 1982) and *Xenopus* muscles (Lappin and Rubin, 1985).

Whether A_{12} AChE is more affected than other forms by innervation in mouse muscles is also unclear. We find that the reduction in A_{12} AChE caused by denervation or *med* is comparable to the reduction in total AChE (Fig. 3*a*; also see Skau and Brimijoin, 1981), whereas Rubin et al. (1985) report a 2-fold greater reduction in A_{12} than in total AChE in TTX-inactivated mouse muscle cultures (but see Inestrosa et al., 1983). In any case, A_{12} AChE in mouse muscle seems approximately as sensitive to innervation as other forms, in contrast to the highly selective effect that innervation has on this form in rat or chicken muscles.

One possibility for the continued presence of A_{12} AChE in denervated or inactive mouse muscles is that preexisting molecules are degraded slowly rather than continuously synthesized. This is unlikely because we observed recovery of all forms in denervated and *med* biceps after AChE was irreversibly blocked with DFP, indicating that the muscle was able to synthesize A_{12} AChE under these conditions. This result differs from that of Rieger et al. (1983), who reported little, if any, synthesis of A_{12} and G_4 AChE in gastrocnemius of *med*^{J} mice after DFP block. The most likely cause of these different results is that we preincubated the muscle in α-bungarotoxin, while Rieger et al. (1983) did not. Kasprzak and Salpeter (1985) have shown that DFP block of AChE causes necrosis of muscle, which delays the recovery of AChE at the neuromuscular junction, and that preincubation of the muscle with α-bungarotoxin prevents the necrosis and delay.

Other AChE forms. Although we see no selective regulation of A_{12} AChE, globular AChE forms, particularly G_4, are selectively regulated in mouse biceps. Indeed, in many mouse fast-twitch muscles, inactivity or denervation reduces this prominent form to negligible levels, and also alters levels of other globular forms (Fig. 1*c*, 4; Skau and Brimijoin, 1981; Gisiger and Stephens, 1983; Rieger et al., 1983). It is unclear to us what functional significance the levels of G_4 AChE have to the muscle, because this form does not appear to be concentrated on the muscle surface in synaptic regions (J. M. Yeakley and C. G. Reiness, unpublished observations), where it would need to be in order to hydrolyze neuronally released ACh.

In the slow-twitch soleus, in contrast to fast-twitch muscles, neither denervation nor inactivity appreciably affects the amount or distribution of globular AChE forms (Fig. 5; Gisiger and Stephens, 1983). The distribution of AChE forms in normal soleus, or in inactivated or denervated soleus (Fig. 5), resembles the distribution of forms seen in denervated or inactive fast muscles (Fig. 4; Gisiger and Stephens, 1983), so it appears that innervation has virtually no effect on AChE in soleus.

Effects of denervation versus med *on AChE forms*

We can use our data to compare the relative effects of denervation and *med*-induced inactivity on AChE in biceps by comparing the ratio of reduction in various AChE forms under the 2 conditions. For example, in advanced (18–21 d) *med* biceps, denervation causes a decrease of G_4 AChE that is 14% as great ($n = 3$) as denervation of normal biceps, i.e., the advanced *med* biceps acts as if it is already 86% denervated by this measure. Estimates derived from reductions of other forms give similar values (79–92% "denervated" character in *med* biceps). Either all fibers retain some residual innervated character, or the muscle contains a mixture of functionally denervated and functionally innervated fibers. Several results support the latter explanation. Weinstein (1980) found that there were 2 classes of fibers, innervated-like and denervated-like, in relatively early stages of the disease in *med*^{J} extensor digitorum longus. This indicates that the onset of the disease is not uniform in all motor units. However, by late stages of the disease, most fibers appear to be affected. In advanced *med* biceps brachii (long head), Duchen and Stefani (1971) found 1/14 fibers that contracted in response to stimulation of the nerve. We find that most (161/184 = 87%) fibers in 18–20 d *med* biceps have unusually low (< -60 mV) resting potentials (A. Akelis and C. G. Reiness, unpublished observations); in normal biceps the resting potential was -71.9 ± 1.0 mV (mean ± SEM; $n = 63$); only 3/63 fibers had resting potentials less than -60 mV (see also Harris and Ward, 1974). Thus, by several measures it appears that 85–90% of the fibers in advanced *med* biceps are affected ("functionally denervated") by the disease.

These results further indicate that the effects of the nerve on AChE in developing mouse biceps are mediated almost exclusively by nerve-induced electromechanical activity of the muscle. Otherwise we would expect to see a less severe effect of *med* than of denervation on AChE, the difference being attributable to activity-independent effects of the nerve in the innervated *med* muscle. In fact, the *med* mutation reduces levels of AChE forms 80–90% as much as denervation, and the difference between them can largely be attributed to the small fraction of fibers that remains functionally innervated in *med* biceps. This allows us to conclude in particular that spontaneously released ACh does not function as a trophic factor (Drachman et al., 1982; see, however, Rochel and Robbins, 1985) for maintenance of AChE in mouse muscle. The frequency and amount of spontaneously released ACh in *med* muscles are the same as in normal muscles (Duchen and Stefani, 1971; Harris and Ward, 1974; Harris and Pollard, 1985), but the amount and distribution of AChE forms are very different in the 2 cases. The small differences between *med* and denervated biceps make it unlikely that the presence of the nerve itself or substances other than ACh are important in regulating AChE, although we cannot exclude the possibility that the failure of evoked release of ACh in *med* nerve terminals prevents release of some trophic factor in adequate amounts (Younkin et al., 1978). However, since we find little difference between innervated and denervated normal soleus, it is clear that neuronally derived factors are not always required to maintain a distinctive pattern of AChE forms in muscles.

Pattern of muscle activity and profile of AChE forms

The observation that innervated soleus has a profile of AChE forms similar to that of denervated and *med* soleus, as well as to denervated and *med* biceps, also indicates that nerve-induced muscle activity is itself not always necessary for maintaining the profile of forms in a muscle. We believe that our results can be explained by the model proposed by Lømo et al. (1985), that the pattern of nerve-induced activity is critical in determining the AChE forms produced by the muscle. We hypothesize that inactive or denervated mouse muscles are intrinsically capable of producing a default pattern of moderate amounts of A_{12} AChE and lesser amounts of other forms (Figs. 2, *b, c*; 4; 5; see also Gisiger and Stephens, 1983). Apparently the tonic activity pattern of the slow-twitch soleus muscle (Lømo et al., 1985) does not alter production of AChE; soleus continues to produce the intrinsic AChE profile. In fast-twitch muscles, superimposition of a phasic pattern of activity on the muscle postnatally seems to increase total AChE, and especially to induce a large increase

in G_4 AChE (Figs. 1, 2). Differences in pattern of activity among various fast-twitch muscles might then account for the slightly different profiles of AChE forms found among them (cf. Gisiger and Stephens, 1983, and Fig. 2). This model appears consistent with the available data concerning the distribution of AChE molecular forms in mouse muscle.

Summary

The *med* mutation has permitted us to distinguish the effects of nerve-induced muscle activity from those of other factors in the development and maintenance of the characteristic profiles of AChE molecular forms in mouse muscles. We find that maturation of the muscle, the presence of innervation, and muscle activity per se do not cause the observed postnatal changes in AChE molecular forms. Our results are thus consistent with the hypothesis that the pattern of muscle activity regulates the amount and proportion of AChE molecular forms produced by the muscle. The *med* system should prove useful in examining the relation between activity and other factors that regulate the properties of the mouse neuromuscular junction during postnatal development.

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