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DENERVATION INCREASES THE DEGRADATION RATE OF ACETYLCHOLINE RECEPTORS AT END-PLATES *IN VIVO* AND *IN VITRO*

BY STUART BEVAN* AND JOE HENRY STEINBACH

*From the Salk Institute, P.O. Box 85800, San Diego, CA 92138, U.S.A., and *Department of Zoology, University College London, Gower Street, London WC1E 6BT*

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SUMMARY

1. We have studied the effect of denervation on the degradation of the existing junctional acetylcholine (ACh) receptors at end-plates in rat muscles. ACh receptors were labelled by injecting animals with iodinated α-bungarotoxin (I-αBT); 1 day later the left hemidiaphragm was denervated. The degradation of bound I-αBT in normal and denervated muscles was examined in organ culture, beginning at various times after denervation *in vivo*.

2. The original, pre-labelled end-plate ACh receptors are degraded more rapidly after denervation. The rate of degradation begins to increase shortly after the nerve is cut and reaches a maximum value at about 9 days of denervation.

3. Muscles denervated only on transfer to organ culture also show an increase in the degradation rate of bound I-αBT with increasing time of denervation (time in culture).

4. In normal diaphragm muscles, the initial rate of degradation of functional ACh receptors, after correcting for non-degradative loss of I-αBT, is 0·0018 h^{-1} ($t_{\frac{1}{2}}$ = 383 h). The maximal rate at denervated end-plates is 0·0073 h^{-1} ($t_{\frac{1}{2}}$ = 94 h). For soleus, sternomastoid, plantaris and intercostal innervated muscles the apparent rate of ACh receptor degradation either *in vitro* or *in vivo* ranged from 0·0005 h^{-1} to 0·002 h^{-1}.

5. The rate of loss of bound I-αBT *in vivo* is more rapid at denervated end-plates than at innervated end-plates. For diaphragm muscles, the rates of I-αBT degradation measured in organ culture are able to describe the relative rates of loss of I-αBT from innervated and denervated muscles *in vivo*.

6. At short times after labelling, a fraction (10–20%) of the I-αBT bound to innervated muscles is degraded more rapidly than the remaining toxin. The possibility that these I-αBT binding sites are degraded at the rate characteristic of extra-junctional receptors on denervated muscle fibres is discussed.

INTRODUCTION

Acetylcholine (ACh) receptors at the neuromuscular junction of adult mammalian skeletal muscle fibres are metabolically quite stable compared with the non-junctional

ACh receptors in denervated muscles. Estimates of the half-life ($t_{\frac{1}{2}}$) of junctional receptors range from 70 to 300 h, while $t_{\frac{1}{2}}$ values of about 20 h have been reported for extrajunctional ACh receptors. Studies of receptor degradation usually employ a radioactively labelled snake toxin, α-bungarotoxin (αBT), to follow the rate of degradation. αBT binds specifically and with high affinity to muscle ACh receptors and the degradation of the toxin to its constituent amino acids appears to accompany that of the liganded receptor (Merlie, Changeux & Gros, 1976; Gardner & Fambrough, 1979).

Chang & Huang (1975) first reported that the amount of tritiated αBT remaining bound to end-plate regions of hemidiaphragm muscles decreased *in vivo* more rapidly for denervated than for innervated muscles. In contrast, Berg & Hall (1975) found no difference in the loss of iodinated αBT (I-αBT) from normal and denervated hemidiaphragms in organ culture, when the muscles had been labelled with I-αBT and one hemidiaphragm denervated 5 days before the start of organ culture. More recently a number of reports have appeared indicating that *in vivo* I-αBT is lost more rapidly from denervated than from innervated end-plates (Brett & Younkin, 1979; Loring & Salpeter, 1980; Levitt, Loring & Salpeter, 1980; Levitt & Salpeter, 1981; Stanley & Drachman, 1981). These authors have concluded that the metabolic stability of the pre-existing ACh receptors at adult end-plates is altered by denervation. In these studies, however, the rates of ACh receptor degradation have been estimated by injecting animals with I-αBT and measuring the amount of radioactivity bound to individual muscles after various survival times. Using this approach it is not possible to determine whether bound I-αBT is actually degraded more rapidly at denervated end-plates, rather than being lost by means other than degradation, nor is it possible to analyse accurately the kinetics of degradation.

A direct demonstration that the degradation rate of junctional ACh receptors is altered by denervation would be of interest for two general reasons. First, it would indicate that the presence of the nerve is required for the maintained metabolic stability of end-plate ACh receptors and that the metabolic stability of these existing receptors may be altered by physiological processes. Secondly, since adult muscle in organ culture is necessarily denervated, it would suggest that studies of junctional receptor metabolism *in vivo* and *in vitro* may not be directly comparable. We have, therefore, used the organ culture technique, which allows the degradation of I-αBT to be measured directly and accurately, to analyse the kinetics of I-αBT degradation at denervated end-plates. We have also examined the degradation of I-αBT bound to end-plates denervated only upon transfer to organ culture.

METHODS

All experiments were made at The Salk Institute using female Sprague–Dawley rats of 160–200 g weight. Data were analysed with the aid of a laboratory computer at The Salk Institute. Purified α-bungarotoxin (a gift of Dr J. Patrick, The Salk Institute) was iodinated and the di-iodinated form (I-αBT) purified according to the methods of Vogel, Sytkowski & Nirenberg (1972). Rats were anaesthetized with ether for all injections and surgical operations. Each animal was injected in the left thoracic cavity with 2×10^{-11} mol of I-αBT in 100 μl of phosphate-buffered saline (I-αBT specific activity, 9×10^9 c.p.m. nmol^{-1}). One day later the left hemidiaphragm was denervated in most animals by removing a 2–3 mm length of phrenic nerve at the point of nerve entry into the muscle. In some animals a 1–2 mm length of the left sciatic nerve was removed in the mid-thigh

at the same time. At various times thereafter rats were killed by exsanguination under ether anaesthesia and the diaphragm, sternomastoid, soleus and, in some cases, plantaris muscles removed. Hemidiaphragm muscles were transferred to organ culture, while the amount of I-αBT bound to the junctional regions of the other muscles was determined immediately.

The duration of the 'pulse' of free I-αBT after intrathoracic injection is not precisely known, but is most likely short. Eighteen to twenty hours after injection the serum concentration of intact I-αBT is less than 2×10^{-10} M, determined from the concentration of radioactivity excluded on P-2 column chromatography (Steinbach, Merlie, Heinemann & Bloch, 1979) or by precipitation with anti-αBT antibody (Steinbach, 1981*a*). Only about 20% of the available I-αBT binding sites are occupied by the injected I-αBT (Merlie, Heinemann & Lindstrom, 1979; Steinbach *et al.* 1979). Finally, more than 95% of the radioactivity bound to a diaphragm muscle removed 18 h after injection of I-αBT is precipitable by anti-ACh receptor antibody (Merlie *et al.* 1979). These results demonstrate that the injected I-αBT pulse is non-saturating and of short duration, and that the muscle-associated I-αBT is bound to ACh receptors.

Determination of junctionally bound I-αBT

Sternomastoid, soleus and plantaris muscles were washed for 1 h in a modified Eagle medium (Vogt & Dulbecco, 1963) buffered to pH 7·2 with 15 mM-HEPES. They were then pinned out and the excess connective tissue removed; this procedure extended the total wash period in medium to about 2 h. The muscles were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline for 1 h at room temperature and then washed for 30 min in medium. During this wash the muscles were teased longitudinally into three to five bundles. End-plates were stained for cholinesterase by the method of Karnovsky & Roots (1964), using a 60 min incubation at room temperature. Muscles were then viewed under a dissecting microscope and cut into cholinesterase-stained (junctional) and unstained (non-junctional) segments. For each muscle the pooled junctional and non-junctional regions were weighed and the amount of radioactivity bound to each of these regions determined. The radioactivity bound to the end-plate-containing segments was corrected, on a wet weight basis, for that bound to the extrajunctional areas and the resultant figure termed 'junctional' or 'end-plate' binding. Pieces of non-muscle tissue (fat, tendon and glands) were washed, fixed and weighed in the same way as the muscles to estimate the degree of non-specific binding. At all times after I-αBT injection, the amount of radioactivity per milligram weight in non-junctional regions of sternomastoid and plantaris muscles was indistinguishable from that in non-muscle tissues. For the first 3 days after injection the non-junctional binding of I-αBT in soleus muscles was found to be higher than in the other muscles examined; this may reflect a higher density of extrajunctional ACh receptors in this muscle.

Organ culture of hemidiaphragm muscles

Hemidiaphragm muscles were cultured using a method described in detail elsewhere (Merlie *et al.* 1979). Briefly, diaphragm muscles were washed for 1–2 h at room temperature in 50–100 ml of modified Eagle medium plus 5% (w/v) new-born calf serum equilibrated with 95% oxygen/5% carbon dioxide (pH 7·2). Adherent connective tissue and as much of the ribs and associated intercostal muscles as possible were removed and the diaphragm divided into two hemidiaphragm–rib portions. Each of these was pinned to a sterilized, sloping (10°) layer of Sylgard (Dow-Corning, Michigan) in a 100 mm diameter glass Petri dish. Ten millilitres of medium plus 5% new-born calf serum was added to each dish, the dishes were placed in a plastic organ culture chamber (Billups-Rothenberg, Del Mar, California) and the chamber gassed with 95% oxygen/5% carbon dioxide. The chamber was moved to a 37 °C room where it was placed on a rocking table modified so that the muscles were immersed in the bathing solution for about 5 s once every 35 s. After 1–2 h the medium was removed and discarded. Ten millilitres of culture medium, which consisted of medium with 5% new-born calf serum, 35 μg ml^{-1} *p*-aminobenzoic acid and 50 μg ml^{-1} bovine pancreatic insulin, was then added to each dish. The dishes were returned to the plastic chamber which was regassed with the oxygen/carbon dioxide mixture and returned to the rocking table in the 37 °C room. At approximately 12 h intervals the entire volume of culture medium was collected and replaced with a fresh 10 ml aliquot.

The amount of radioactivity in each aliquot of culture medium was measured with a γ-counter. At the end of the culture period the tissue was divided into a hemidiaphragm segment and a segment containing the attached ribs and intercostal muscles, and the amount of radioactivity in each was

determined. The total radioactivity initially bound to the cultured tissue was calculated and curves constructed for the percentage of radioactivity remaining bound to the tissue as a function of time in culture. We shall refer to such curves as release curves.

Release of radioactivity from intercostal muscles

The release curves for the combined hemidiaphragm–intercostal muscle cultures were the starting point for subsequent data analysis. Since only the hemidiaphragm was denervated and not the intercostal muscles in the attached ribs, we estimated the contribution of the intercostal muscle

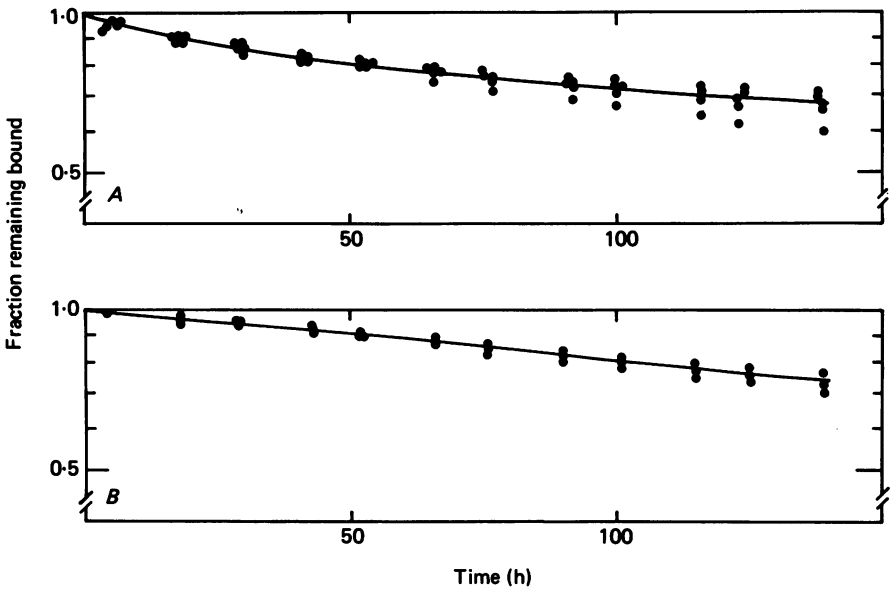


Fig. 1. The fraction of I-αBT remaining bound to intercostal muscles, plotted semi-logarithmically, is shown as a function of time in culture. The symbols show values for individual cultures (five in panel *A*, three in panel *B*), while the lines show interpolated mean values as a function of time. Cultures were started 1 day (panel *A*) or 15 days (panel *B*) after injection with I-αBT. The mean values were taken as the averaged intercostal muscle release for subtraction from data from hemidiaphragm–intercostal muscle cultures (see text). The coefficient of variability (S.D./mean; c.v.) at 100 h for intercostal muscle cultures ranged from 0·027 to 0·048; for the curve in panel *A* it was 0·048 while for the curve in panel *B* it was 0·028.

The release of radioactivity from cultures of intercostal muscles showed the same pattern as release from hemidiaphragms (see Results). When cultures were prepared from animals at short times after I-αBT injection, the initial rate of loss of radioactivity was more rapid than for cultures prepared at longer times after injection, as can be seen by comparing the initial portions of the curves in panel *A* and panel *B*. In cultures prepared at long times after injection of I-αBT, the rate of release increased during the culture period as shown by the downward curvature in panel *B*.

to the total release curve for the combined tissues. Portions of rib cage were cut to a size comparable to that normally attached to the dissected hemidiaphragm muscle and then cultured as described above.

Release curves for intercostal muscle cultures were quite reproducible for multiple samples from the same animal and also for tissue from different animals at equivalent times after I-αBT injection (Fig. 1*A*, *B*). The release curves for cultured intercostal and hemidiaphragm–intercostal muscles showed similar changes as a function of time after injection (see Results), so averaged intercostal release curves were computed for tissue removed from animals 1, 3–4, 7–9, 11–12 and 15 days after

I-αBT injection. The time course of I-αBT release from the hemidiaphragm was estimated using the release curves for the hemidiaphragm–intercostal muscle cultures and the appropriate averaged intercostal muscle release curve. For this, the radioactivity remaining in the rib portion of a hemidiaphragm–intercostal muscle culture at the end of the culture period was determined and the appropriate averaged intercostal muscle release curve scaled to this value. The calculated release from the intercostal muscle was then subtracted from the hemidiaphragm–intercostal muscle release curve to yield a release curve for the hemidiaphragm alone. This correction was negligible if, at the end of the culture period, the amount of radioactivity bound to the rib segment was less than 20% of that bound initially to the cultured tissues. Fig. 2*A* and *B* show data in which a larger proportion of the total radioactivity was associated with the intercostal muscles. Under these conditions the corrected release curve for the normal hemidiaphragm was very similar to the original release curve (Fig. 2*B*). In contrast, the release curve for the denervated half of the diaphragm was more affected by the correction for the intercostal muscle contribution (Fig. 2*A*). The use of this correction procedure assumes that radioactivity is released from the intercostal muscles at the same rate whether they are cultured alone or with attached diaphragms. Although this assumption appears to be reasonable we have no independent evidence for its validity.

Column chromatography

To distinguish between radioactivity released by I-αBT dissociation and by degradation, aliquots of culture medium were analysed by gel exclusion chromatography using Biorad P-2 resin beads. The fraction of released radioactivity which was of low molecular weight (*F*) was used as a measure of degraded I-αBT (cf. Merlie *et al.* 1979).

RESULTS

Our basic observation is illustrated in Figs. 2 and 3, which show the loss of bound radioactivity from cultured muscles as a function of time in culture. The original end-plate ACh receptors, which were labelled with I-αBT before denervation, are degraded more rapidly at end-plates denervated *in vivo* than at normal end-plates. For every pair of denervated and contralateral normal hemidiaphragm cultures, radioactivity was lost more rapidly from the denervated side. This was apparent even before correcting the release curves for the contribution of the intercostal muscles, as can be seen by comparing the upper curve in Fig. 3*A* (normal hemidiaphragms cultured 4 or more days after I-αBT injection) with the curves in Fig. 3*B* for denervated hemidiaphragms cultured at comparable times after injection.

Fig. 3*A* demonstrates that for normal muscles radioactivity is lost more rapidly from cultures initiated at short times after I-αBT injection (lower curve) than from muscles cultured at longer times after injection (upper curve). The downward curvature of the upper line in Fig. 3*A* also shows that there is an acceleration in the rate of release of radioactivity from normal muscles during the culture period when the muscles are transferred to organ culture more than 4 days after injection with I-αBT.

The major portion of the radioactivity released from the denervated muscles was of low molecular weight as judged by gel exclusion chromatography (Fig. 4). Comparison of the data shown in Fig. 4*A* (denervated muscles) and Fig. 4*B* (normal muscles) shows that the fraction of released radioactivity which represents degraded I-αBT is larger for denervated than for normal muscles. Furthermore, the loss of radioactivity was greatly reduced when cellular metabolism was inhibited by addition of 5 mM-sodium fluoride and 2 mM-dinitrophenol to the culture medium (Fig. 5). These two observations indicate that the increased loss of radioactivity from denervated muscles reflects an increased rate of cellular degradation of bound I-αBT

and, presumably, an increased rate of degradation of the ACh receptors to which the I-αBT is bound.

Inspection of Fig. 4*B* shows that the fraction of radioactive material of low molecular weight (*F*) released from normal muscles is quite consistent between cultures. In addition, values of *F* are similar at given times in culture irrespective

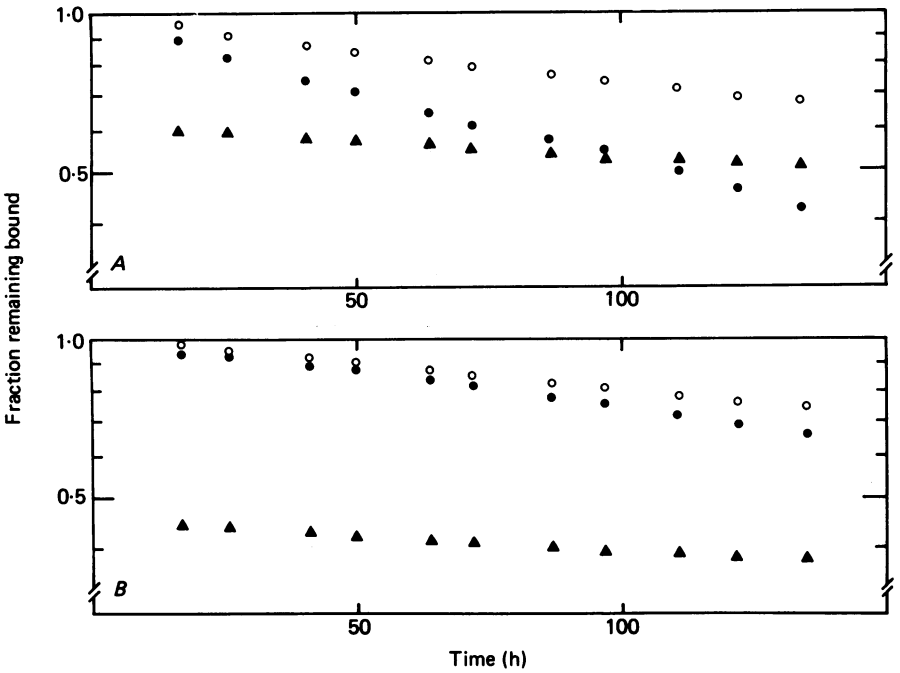


Fig. 2. This Figure illustrates how release curves for hemidiaphragm muscles were obtained from the total release curve for hemidiaphragm–intercostal muscle cultures. Panel *A* shows a release curve for a hemidiaphragm–intercostal muscle culture in which the hemidiaphragm had been denervated *in vivo* for 10 days before the start of organ culture. Panel *B* shows the release curve for the contralateral normal hemidiaphragm–intercostal muscle. The open circles in panels *A* and *B* show the total release curves for the two preparations, while the filled triangles show the calculated fraction contributed by the intercostal muscles (see text). The filled circles show the calculated release curves for the hemidiaphragm muscles alone (hemidiaphragm release curve), renormalized to a value of 1 at the start of the culture period. These data were chosen because a large fraction of the total radioactivity at the end of the experiment was associated with the intercostal muscles (52% in the case shown in panel *A* and 38% in panel *B*) and so the correction for the intercostal muscles was maximal. The median fraction for innervated muscle cultures was 18% (range 7–51%) while for denervated hemidiaphragm–intercostal muscle cultures it was 19% (9–52%).

of the time between I-αBT injection and the start of organ culture. Fig. 4*B* also shows that *F* is similar for intercostal muscle cultures (open symbols) and for hemidiaphragm–intercostal muscle cultures (filled symbols). The lines through the points show interpolated values of *F* which were used to calculate the rates of loss of bound I-αBT through degradative and non-degradative processes. A further point illustrated in Fig. 4 is that *F* was often lower in the first few hours after transferring

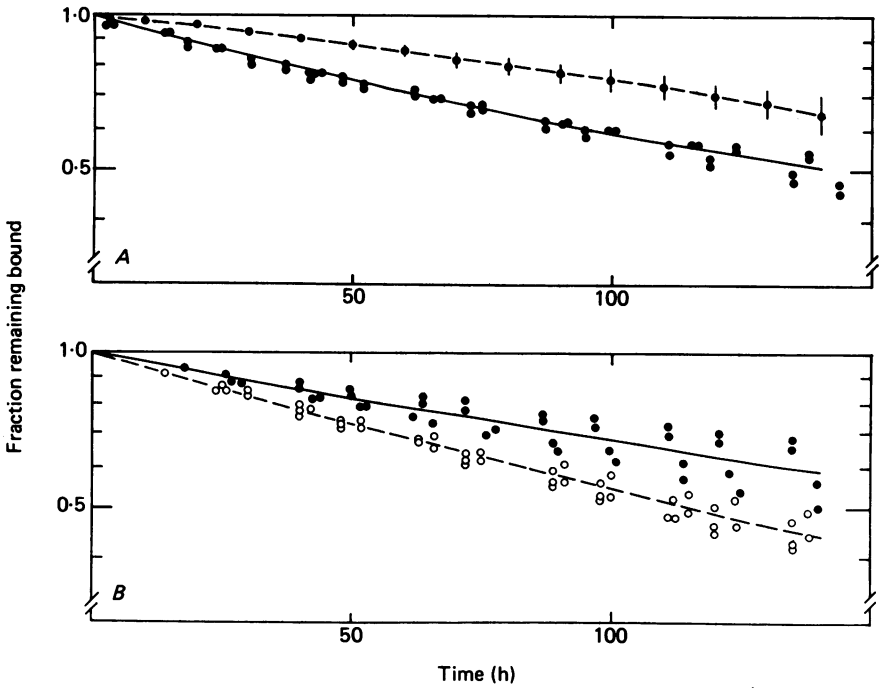


Fig. 3. Release curves for normal hemidiaphragm–intercostal muscle cultures and denervated hemidiaphragm–intercostal muscle cultures. The lower curve in panel *A* shows the calculated fraction of the initial radioactivity remaining bound to 4 normal hemidiaphragm–intercostal muscle cultures prepared 1 day after injection of I-αBT (the line through the points shows the mean for these data). The upper dashed curve in panel *A* shows the mean (±1 S.D. at 10 h intervals) for twenty-two normal hemidiaphragm–intercostal muscle cultures prepared 4 days or longer after injection of I-αBT (252 time points, not shown in Figure). Panel *B* shows release curves for denervated hemidiaphragm–intercostal muscle cultures prepared 7 or 8 days after denervation (open circles, five cultures) and 10 or 11 days after denervation (filled circles, four cultures); the lines through the points show calculated mean values. The coefficient of variability (c.v.) after 100 h in culture ranged from <0·01 to 0·06 for normal hemidiaphragm–intercostal muscle cultures (in panel *A*, for the upper curve the c.v. at 100 h was 0·0520; for the lower curve it was 0·0355). Denervated hemidiaphragm–intercostal muscle cultures showed more variability; the c.v. after 100 h in culture ranged from 0·04 to 0·26 (in panel *B*, for the upper curve the c.v. at 100 h it was 0·098; for the lower curve it was 0·060). The increased variability in denervated muscle cultures is expected for two reasons: first, since the mean amount bound declines more rapidly the c.v. should be larger at a given time; and secondly, because the contribution of the intercostal muscles to the release curves is larger and differs from one culture to another.

muscles to organ culture, irrespective of the time between I-αBT injection and muscle dissection. We do not know the reason for this observation.

Estimation of I-αBT degradation rates

The first step in our analysis was to estimate the initial rates of loss of bound radioactivity from cultured hemidiaphragm muscles. Release curves for the hemidiaphragm muscle were constructed by subtracting the contribution of the intercostal muscles from the combined hemidiaphragm–intercostal muscle release curves. To

estimate the initial rate of loss of bound I-αBT, straight lines were fitted to semilogarithmic transforms of the data for the first 50 h of culture. The slope of each line was taken as an estimate of the initial rate of I-αBT loss, giving the values shown in Fig. 6. Several points emerge on inspection of this Figure. First, the initial rate

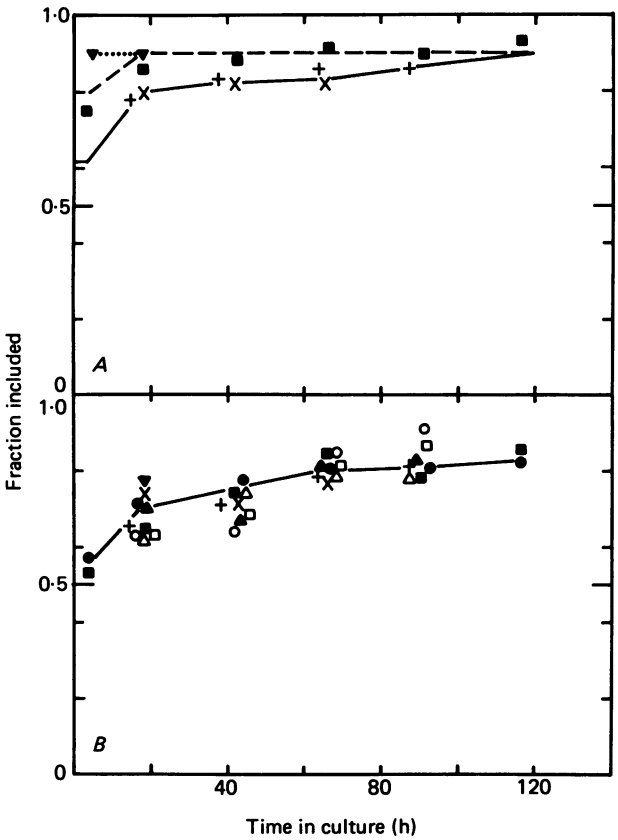


Fig. 4. The fraction of released radioactivity which is of low molecular weight is shown as a function of time in organ culture. The fraction of the released radioactivity in the included volume on P-2 column chromatography (*F*) is plotted as a function of the time at which the culture medium sample was collected from cultures of denervated hemidiaphragm–intercostal muscles (panel *A*), normal hemidiaphragm–intercostal muscles (filled symbols, panel *B*) and intercostal muscles (open symbols, panel *B*). The different symbols in panel *A* indicate that cultures were initiated after different periods of denervation *in vivo* (+, 2 days; ×, 5 days; ■, 8 days; ▼, 11 days). The symbols in panel *B* indicate cultures started at various times after injection of I-αBT (●, 1 day; +, 3 days; ▲, 4 days; ×, 6 days; ■, 9 days; ▼, 12 days).

of loss was greater for denervated than for normal hemidiaphragms. Secondly, when paired muscles from unoperated animals were studied, the initial rates were similar for the two sides. Thirdly, the rates of loss were similar for hemidiaphragms from normal unoperated animals and for hemidiaphragms contralateral to denervated muscles. For this reason, results from these latter two groups of hemidiaphragms will be analysed together in the rest of this paper. Finally, during the first few days after

I-αBT injection the initial rate of loss of radioactivity from normal muscles appeared to decrease. This observation will be discussed in a subsequent section of the paper.

Kinetic analysis of release curves

The analysis presented in this section was made on the release curves for hemidiaphragm muscles after correction for release from the intercostal muscles. The analysis was restricted to cultures started more than 4 days after injection with I-αBT, to avoid the complication of the change in the initial release rate at earlier times after injection.

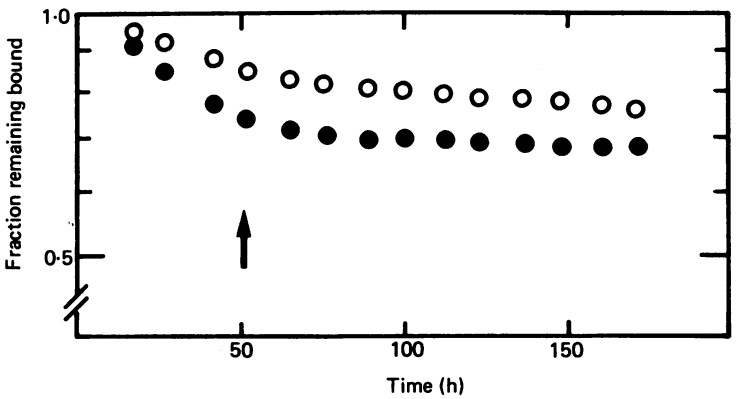


Fig. 5. Metabolic inhibitors decrease the loss of radioactivity from cultured muscle. Release curves are shown in a semilogarithmic plot for a denervated (12 days) hemidiaphragm–intercostal muscle culture (filled symbols) and the contralateral normal muscle culture (open symbols). At the arrow (48 h after start of culture) normal culture medium was replaced with complete culture medium containing 5 mM-sodium fluoride and 2 mM-dinitrophenol.

The first stage in the analysis was to calculate apparent first-order release rates (r) for each successive pair of points in the release curve. This calculation was made using values for the amount of radioactivity bound to the muscle at the start of each collection period (A_0) and the amount released (R) during that period (t). The relationship is given by the equation $r = -\ln[(A_0-R)/A_0]/t$. There were two reasons for this approach. First, if time-dependent changes in the degradation rate occur, then this is the appropriate approach to reveal and analyse the changes. Secondly, it is straightforward to separate the degradative and non-degradative components of the release process when rate constants are used. To do this, it was assumed that the rate (r) reflected the action of two independent processes acting on the same pool of bound I-αBT molecules: degradation, with rate k, and non-degradative loss, with rate l. It was further assumed that these rates did not change significantly over any one collection period. Under these assumptions, the degradation rate (k) is equal to the calculated release rate (r) multiplied by the fraction of released material which is degraded (F): $k = r \times F$. The value of F was estimated using the lines shown in Fig. 4.

Analysis of the release curves for normal hemidiaphragms (Fig. 7*A*), which were denervated only upon transfer to organ culture, revealed an increase in the rate of

degradation of bound I-αBT with increasing time in culture. For example, over the first 20 h in culture the mean degradation rate (k) is $0{\cdot}0018 \pm 0{\cdot}0004$ h^{-1} (mean $\pm$ S.D. of thirty-nine rates), while between 90 and 110 h k is $0{\cdot}0034 \pm 0{\cdot}0007$ h^{-1} ($n = 35$). The increase in k shown in Fig. 7*A* is statistically significant, judged either by comparing mean rates at the start and end of the culture period (t test, $P < 0{\cdot}001$) or from the regression of k on time in culture (b differs from 0 at $P < 0{\cdot}001$).

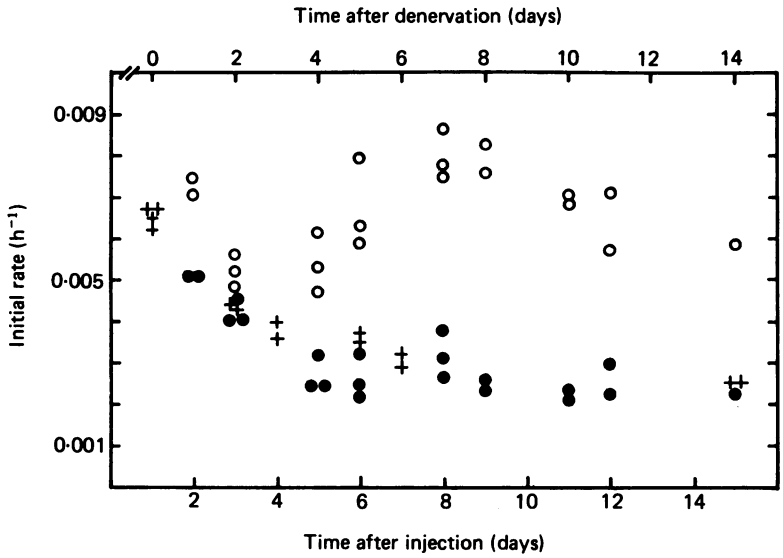


Fig. 6. The calculated initial rate for the loss of radioactivity changes with time. Straight lines were fitted by the method of least squares to semilogarithmic plots of the first 50 h of hemidiaphragm release curves for normal muscles (crosses), denervated muscles (open circles) and normal muscles contralateral to denervated muscles (filled circles). The negative of the slope of the fitted line is plotted against the time of initiation of organ culture. The lower abscissa shows the number of days after injection of I-αBT before the start of culture (all symbols), while the upper abscissa shows the number of days of denervation *in vivo* before the start of culture (open circles).

Fig. 7*B* shows a similar analysis of the release curves for muscles labelled with I-αBT and then denervated for various lengths of time before culturing. Here the rate of degradation increased significantly ($P < 0{\cdot}001$) between 120 h and 200–230 h after denervation, when k reached a peak value of $0{\cdot}0073 \pm 0{\cdot}0016$ h^{-1} ($n = 32$). With longer periods of denervation, the degradation rate declines from this peak level and by 400 h k is significantly less than at 200–230 h ($P < 0{\cdot}001$). The degradation rates for muscles denervated *in vitro* (normal muscles), which are shown in Fig. 7*C* by open circles connected with dashed lines, overlap with those for muscles denervated *in vivo*. This indicates that denervation has similar effects on the rate of degradation of the original end-plate ACh receptors irrespective of whether the muscle is denervated *in vivo* or *in vitro*.

If our kinetic analysis of I-αBT loss from normal and denervated muscles is correct and the degradation rates observed *in vitro* are applicable to the loss of I-αBT *in vivo*, then we should be able to predict accurately the relative number of pre-labelled ACh

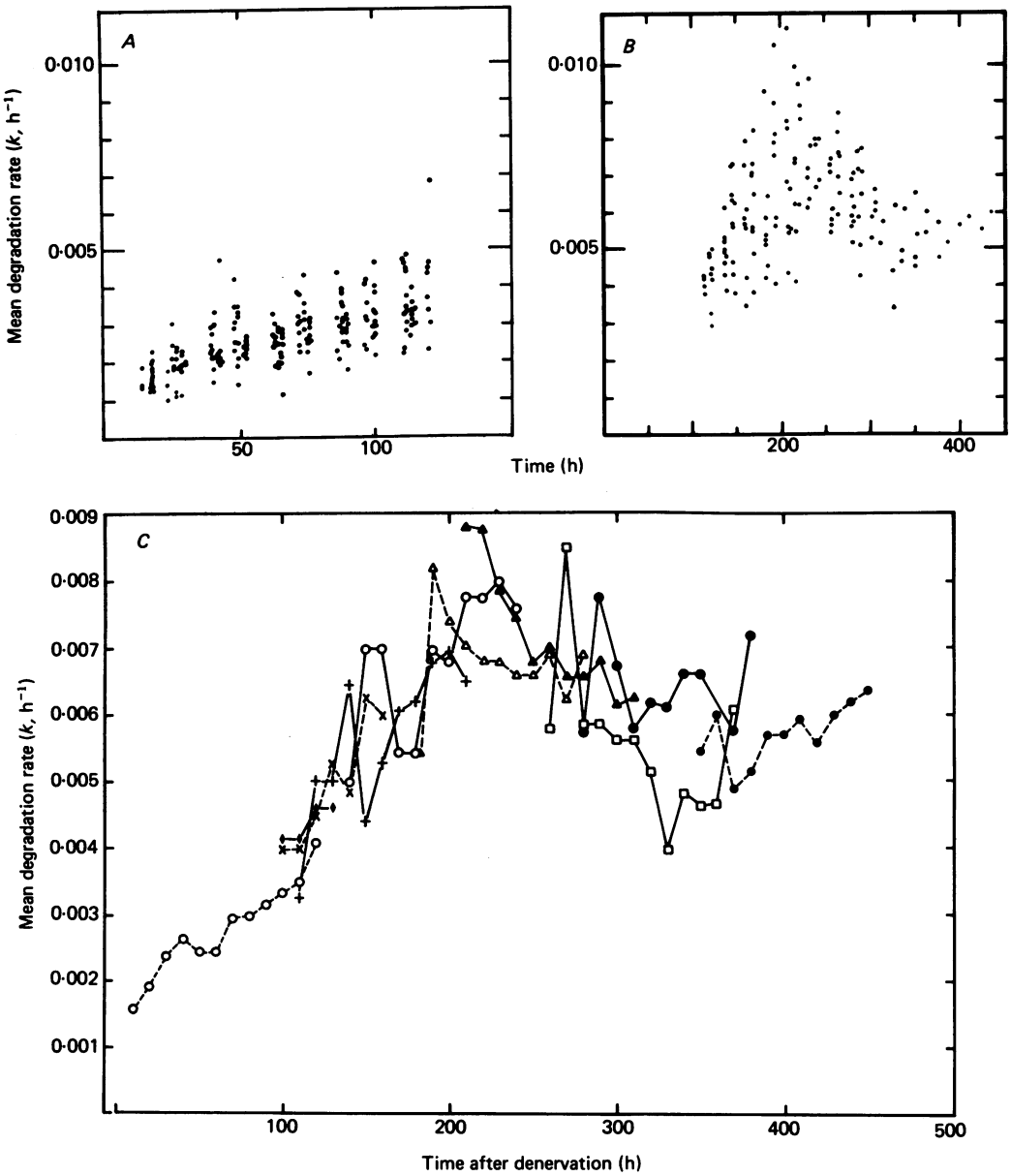


Fig. 7. The estimated rate of degradation of bound I-αBT at various times after denervation. The rate of degradation of bound I-αBT (k) was calculated for each sample collection interval in culture. The rates in panel *A* are shown for normal diaphragm muscle as a function of time in organ culture; calculated rates are plotted as points at the end of each collection interval. All normal muscles were removed from animals 96 h or longer after injection of I-αBT. Panel *B* shows rates calculated for muscles denervated *in vivo*. The total period of denervation (*in vivo* and *in vitro*) is plotted on the abscissa. Panel *C* shows the mean rate (at 10 h intervals) for groups of muscles started in culture at the same time after denervation. Open circles connected with dashed lines show data for normal muscles; other symbols show data from muscles denervated *in vivo* before the start of organ culture. In all cases only data obtained more than 96 h after the injection of I-αBT are shown. In panel *C* the same data are shown as in panels *A* and *B*, but rather than showing individual rates, mean rates for cultures of muscles denervated for the same periods are illustrated.

receptors at innervated and denervated end-plates after various periods of denervation *in vivo*. Fig. 8 shows the results of such an analysis. Here the ratio of the amount of radioactivity bound to a denervated hemidiaphragm divided by that bound to the contralateral innervated muscle is shown as a function of time after denervation. The dashed line, which fits the data reasonably well, represents the predicted ratios. These were calculated by assuming that the mean degradation rates shown in Fig. 7 *C* describe the rates for the original receptors at denervated end-plates, while the degradation rate at innervated end-plates is constant at 0·0018 h^{-1}.

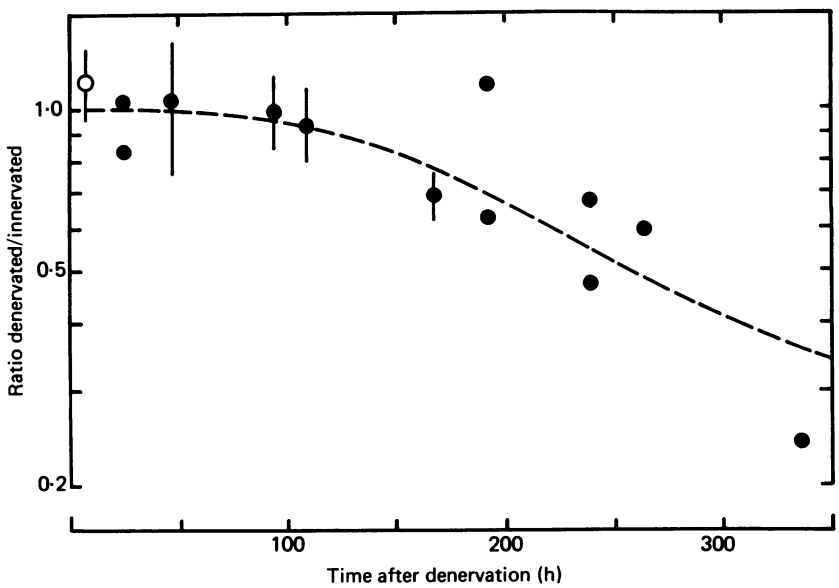


Fig. 8. The ratio of radioactivity bound to denervated hemidiaphragms to that bound to contralateral hemidiaphragms is shown as a function of time after denervation. The total radioactivity bound to denervated and to contralateral hemidiaphragm muscles at the start of organ culture was calculated (see Methods). Symbols show mean ± S.D. of these ratios, or individual values if less than three pairs of muscles were studied. The open symbol near the left ordinate shows the ratio for five pairs of muscles from unoperated animals. The dashed line shows the ratio predicted assuming that the mean degradation rates shown in Fig. 7 describe the rate of degradation of pre-existing receptors at denervated end-plates, while the rate of degradation at innervated junctions is constant at 0·0018 h^{-1}. The predicted ratios are not altered if non-degradative loss occurs at the same rate in the normal and denervated hemidiaphragms.

I-αBT is degraded more rapidly at short times after injection

Our data show that bound radioactivity is lost more rapidly from normal, innervated muscles placed in organ culture when the release is examined at short times after injecting rats with I-αBT (Fig. 6). Analysis of the data indicated that the increased rate of loss was not due solely to the release of high-molecular-weight material but resulted in part from a more rapid rate of degradation. A possible explanation of this observation is that innervated adult mammalian muscle contains some non-junctional ACh receptors, which are degraded more rapidly than junctional

receptors. The presence of such receptors would contribute an initial, rapidly decaying phase to the I-αBT degradation curve. The following analysis was performed to determine whether or not such an explanation is reasonable.

We assume that some fraction (J) of the I-αBT binding sites were degraded *in vitro* at the rates shown in Fig. 7*A*, while the remaining sites ($1-J$) were degraded at the rapid rate found for non-junctional receptors in mammalian muscles ($k = 0{\cdot}032\ h^{-1}$). We calculated the degradation rate for I-αBT bound to muscles removed from animals less than 4 days after I-αBT injection, using the methods described in the

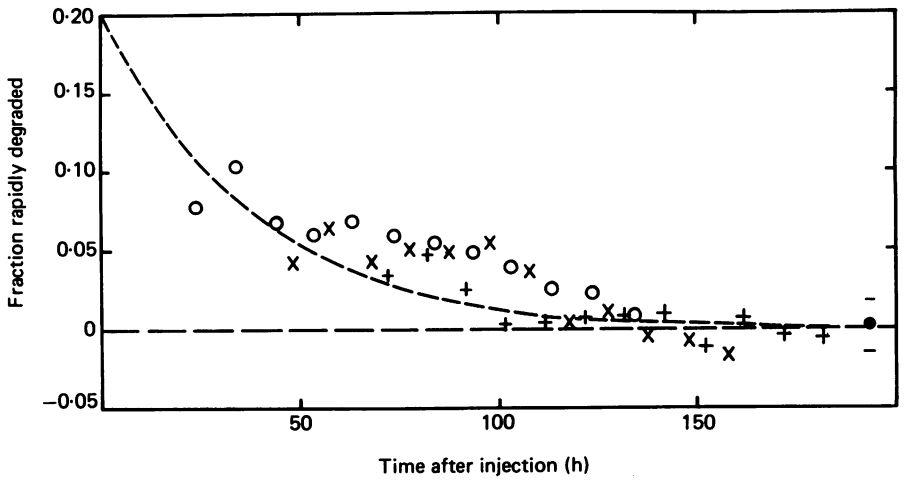


Fig. 9. The calculated fraction of ACh receptors in normal diaphragm which are rapidly degraded at different times after labelling with I-αBT. Release curves for normal hemidiaphragm muscles placed in culture shortly after injection of I-αBT were analysed to estimate the fraction of the bound I-αBT which was rapidly degraded. The different symbols show the means calculated at 10 h intervals in culture for muscles labelled *in vivo* 1 day (○), 2 days (×) and 3 days (+) before being placed in culture. The filled circle on the right shows the mean and range of the rapidly degraded fraction for muscles removed 4 days, 5 days and 6 days after injection. The dashed curve is the curve calculated assuming 19% of the total receptor content of the muscle at the time of injection was of a rapidly degraded type.

preceding section. Then we determined for each calculated rate what fraction of the bound I-αBT would have to have been degraded at the rapid rate to produce the data. The results of these calculations are shown in Fig. 9. There is fairly good agreement between the data points and the predicted curve if approximately 20% of the ACh receptors labelled with I-αBT are degraded at the rapid non-junctional rate.

Non-degradative loss of I-αBT from hemidiaphragm muscles

The rate of non-degradative loss (l) of I-αBT from normal and denervated end-plates in culture can be estimated in the same way as the rate for degradation. To calculate this rate we used the formula $l = r \times (1-F)$. After the first day in culture l was approximately $0{\cdot}0008\ h^{-1}$ for both normal ($l = 0{\cdot}00077 \pm 0{\cdot}0002\ h^{-1}$, 142 rates) and denervated ($l = 0{\cdot}00085 \pm 0{\cdot}00026\ h^{-1}$, 73 rates) hemidiaphragm–intercostal muscle cultures. This estimate is close to the two estimates for the rate of release of

radioactivity from muscles poisoned with fluoride and dinitrophenol shown in Fig. 5 (0·0005 and 0·0008 h^{-1}).

Estimates of the degradation rates of end-plate receptors in other muscles

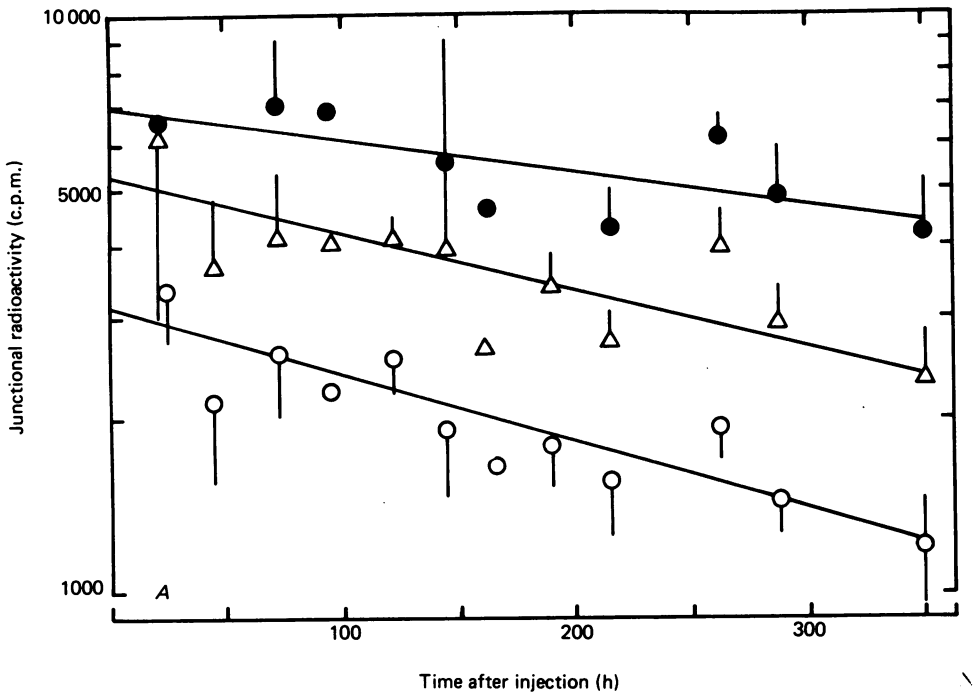
The release of radioactivity from organ-cultured intercostal muscles was analysed in the same way as release from hemidiaphragm muscles. The results qualitatively resemble those described above for the hemidiaphragm. The rate of I-αBT degradation (k) was initially low for muscles removed from rats more than 4 days after I-αBT injection and increased with time in culture. Over the first 24 h in culture k was $0{\cdot}0011 \pm 0{\cdot}0005$ h^{-1} ($n = 18$), increasing to $0{\cdot}0019 \pm 0{\cdot}0005$ h^{-1} ($n = 18$) between 70 and 90 h in culture. The rate of degradation was greater in muscles removed at earlier times (1, 3 or 4 days) after injection. Results analogous to those in Fig. 9 were calculated, but for the intercostal muscle the data were consistent with the hypothesis that only 10% of the originally bound I-αBT was degraded at a rate of 0·032 h^{-1}.

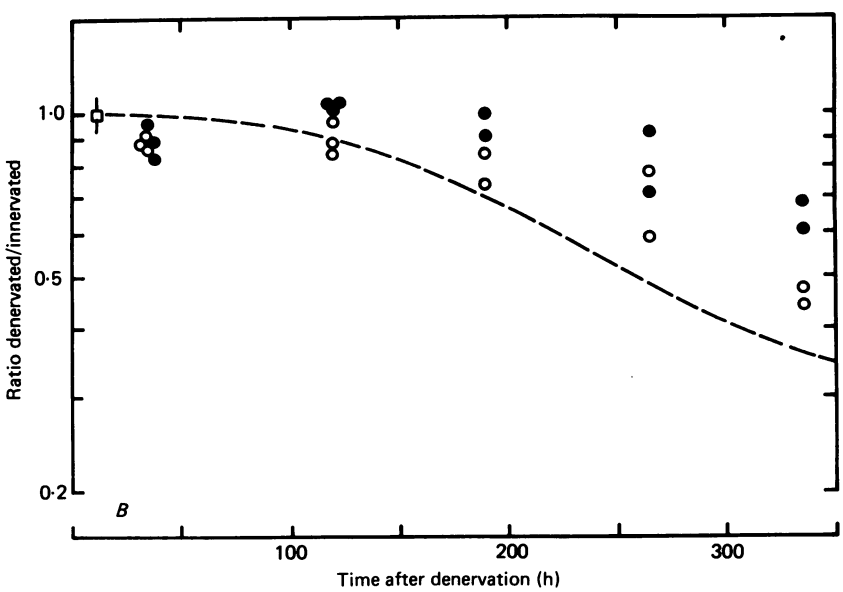
We also examined the rate of loss *in vivo* of bound I-αBT from other muscles. Rats were injected with I-αBT and after various survival times the sternomastoid, soleus and, in some rats, plantaris muscles were removed. The amount of radioactivity bound to the end-plate regions of these muscles was then determined. For innervated muscles, we found that the level of I-αBT bound to the end-plate regions decreased slowly with time, giving rates of loss of between 0·001 and 0·003 h^{-1} (Fig. 10*A*). When plantaris and soleus muscles were denervated 1 day after injection, radioactivity was lost from the end-plate zones more rapidly for the denervated muscles than for the contralateral innervated muscles (Fig. 10*B*). The ratio of the counts bound to the denervated side divided by the counts bound to the innervated side decreased more slowly than the ratio for the hemidiaphragm, but qualitatively the results for diaphragm, soleus and plantaris muscles were similar.

DISCUSSION

Our observations show that ACh receptors at adult innervated rat diaphragm end-plates are degraded at a slow rate (0·0018 h^{-1}; $t_{\frac{1}{2}} = 383$ h). After denervation the rate of degradation of these receptors changes, increasing over 9 days to a peak rate of 0·0073 h^{-1} ($t_{\frac{1}{2}} = 94$ h). This rate is less than that reported for receptors in non-junctional regions of denervated muscles (0·032 h^{-1}; $t_{\frac{1}{2}} = 22$ h). Our data on other

Fig. 10. Loss of radioactivity from end-plate regions of muscle *in vivo*. Panel *A* shows the radioactivity bound to end-plate regions of innervated muscles at various times after injection of I-αBT. Symbols show mean ± S.D. or individual values if fewer than three animals were studied (sternomastoid muscles, open triangles; soleus muscles, open circles; plantaris muscles, filled circles). The data are plotted semilogarithmically; the straight lines have been fitted using a linear least squares procedure. The slope of each line is significantly different from zero (t test, $P < 0{\cdot}025$. The slopes and 95% confidence limits are: sternomastoid, $0{\cdot}0023 \pm 0{\cdot}0008$ h^{-1}; soleus, $0{\cdot}0027 \pm 0{\cdot}0008$ h^{-1}; plantaris, $0{\cdot}0013 \pm 0{\cdot}0012$ h^{-1}. The radioactivity has been corrected for the decline in specific activity of the radio-iodine. Panel *B* shows the ratio of counts bound to denervated end-plate regions over those bound to contralateral innervated end-plate regions for soleus (open circles) and plantaris (filled circles) muscles, as a function of time after denervation. The dashed line is the calculated curve shown in Fig. 8. The open square shows the ratio of counts bound to pairs of normal muscles (mean ± S.D.; $n = 15$).


10 000
5000
1000
Junctional radioactivity (c.p.m.)
A
100
200
300
Time after injection (h)


1·0
0·5
0·2
Ratio denervated/innervated
B
100
200
300
Time after denervation (h)

rat muscles are qualitatively in agreement with this finding, although there may be some quantitative difference between different muscles. We conclude that the metabolic stability of existing junctional ACh receptors may be altered by physiological events following denervation. This observation is to be distinguished from the finding that newly synthesized receptors which are inserted into denervated end-plate regions have the short half-life characteristic of extrajunctional ACh receptors (Levitt & Salpeter, 1981).

Conclusions similar to ours have recently been reported for muscles *in vivo* on the basis of the rate of loss of bound I-αBT from end-plates after denervation (Chang & Huang, 1975; Brett & Younkin, 1980; Loring & Salpeter, 1980; Levitt & Salpeter, 1981; Stanley & Drachman, 1981). Our experiments, however, demonstrate for the first time that the increased rate of loss of I-αBT results from an increased rate of degradation of previously existing end-plate ACh receptors. In addition, using the organ culture technique, we have been able to describe the time course of the change in degradation rate. There are many possible ways in which our kinetic data could be produced, of which the two clearest alternatives are as follows. The first is that all previously junctional receptors are equally affected by denervation and have a metabolic stability which changes with time as shown in Fig. 7. The other alternative is that after denervation some fraction of the original junctional receptors is converted to a less stable form – perhaps a non-junctional form. The curve in Fig. 7, in this case, is the result of the changing rate of conversion of the receptor pool after denervation. Our data cannot distinguish between these alternatives.

It is not known whether an increase in degradation rate after denervation is unique to pre-existing junctional ACh receptors, or whether the end-plate as a whole is metabolized more rapidly. It has been reported that the 16 S, end-plate-specific form of acetylcholinesterase is rapidly lost after denervation (Vigny, Koenig & Rieger, 1976; Weinberg & Hall, 1979).

It is known that the total number of ACh receptors aggregated at denervated end-plates decreases only slowly after denervation (Frank, Gautvik & Sommerschild, 1975; Steinbach, 1981*b*) – far more slowly than the rate at which the original junctional ACh receptors disappear (cf. Fig. 8). It appears, therefore, that even at established adult end-plates receptor density and receptor metabolism are not tightly coupled. This is consistent with previous observations that during development receptor aggregation, receptor function and receptor metabolism appear to be independently controlled (Steinbach *et al.* 1979; Fischbach & Schuetze, 1980; Reiness & Weinberg, 1981; Michler & Sakmann, 1980; Steinbach, 1981*a*).

The mechanism by which the metabolic stability of ACh receptors is altered after denervation is not known. We have found, however, that mild collagenase digestion of rat diaphragm muscles increases the rate of degradation of junctional receptors but not of extrajunctional receptors (R. Bloch, S. F. Heinemann, J. P. Merlie & J. H. Steinbach, unpublished observations). Results obtained by others have implicated the basal lamina in the aggregation of ACh receptors at end-plates (Burden, Sargent & McMahan, 1979; Bader, 1981). It is possible that molecules in the basal lamina, which are removed or modified by collagenase treatment, also play a role in determining the metabolic stability of junctional ACh receptors; these molecules may be sensitive to enzymes released at end-plates after denervation.

Our measurements of ACh receptor degradation rates show that the rate peaks at about 9 days after denervation; thereafter the calculated rate shows a significant decrease. Such a slowing in the degradation rate should be reflected in the total number of original end-plate ACh receptors remaining at long times after denervation. Unfortunately, our data on the total number of remaining end-plate receptors do not cover a sufficiently long period after denervation to confirm the kinetic data. Levitt & Salpeter (1981) have measured the total number of original receptors at end-plates at long times after denervation and have found that the relative number at denervated versus innervated end-plates stops decreasing after about 2 weeks after denervation.

Junctional ACh receptors are normally quite stable metabolically. For five different rat muscles, our estimates for degradation rates range from less than 0·001 h^{-1} to 0·002 h^{-1}, after correcting for a non-degradative loss rate of 0·0008 h^{-1}. These values agree well with several other estimates for end-plate ACh receptor stability (Linden & Fambrough, 1979; Michler & Sakmann, 1980; Reiness & Weinberg, 1981). Our rates are rather less than previous estimates for innervated rat diaphragm muscles placed in organ culture at fairly short times after labelling with I-αBT (for example 0·005 h^{-1}: Steinbach *et al.* 1979). We feel that this disagreement is due, in large part, to the fact that the rate depends on the interval between labelling with I-αBT and the start of organ culture.

Although we do not know the basis for the rapid phase of I-αBT loss at short times after labelling, we demonstrated in the Results section that the change in the degradation rate with time after injection of I-αBT is consistent with the hypothesis that approximately 20 % of the ACh receptors in a normal rat diaphragm are degraded at the rate characteristic of extrajunctional receptors. We have also re-analysed similar release data for twenty-nine additional hemidiaphragms, which have been published elsewhere (Steinbach *et al.* 1979), with similar results. The median proportion of rapidly degraded receptors was 28 % with a range of 9 % to 45 %. There are approximately $2·5 \times 10^7$ ACh receptors per hemidiaphragm end-plate in rats of this size (Fambrough & Hartzell, 1972; Steinbach, 1981*a*), so approximately 5×10^6 rapidly degraded receptors would be present per muscle fibre. This number corresponds to a density of about five receptors per square micrometre of muscle membrane, if we assume that these receptors are evenly distributed over a muscle fibre of 40 μm diameter (Fambrough, 1974) and 1 cm length. The density of extrajunctional receptors on muscle fibres from innervated diaphragms has been reported to be less than six per square micrometre (Hartzell & Fambrough, 1972; Fambrough, 1974), which is in approximate agreement with our hypothesized density. It is, therefore, possible that 20 % of the receptors in normal diaphragm muscles are not only rapidly degraded but also extrajunctionally located. In agreement with our observations, Linden & Fambrough (1979) have found that approximately 10 % of the I-αBT molecules bound to normal rat extensor digitorum longus muscles are degraded at a more rapid rate than the remaining 90 %. Iso-electric focusing of ACh receptors extracted from innervated rat hind limb muscles has also shown the presence of some I-αBT binding material at a iso-electric point characteristic of non-junctional ACh receptors, with the estimated proportion being less than 25 % of the total extracted binding sites (Nathanson & Hall, 1979). In sum, our data are consistent with the

simple hypothesis that a proportion of ACh receptors in innervated rat muscles is degraded at the rate typical of non-junctional receptors; these rapidly degraded receptors could be located extrajunctionally. We cannot, however, exclude a variety of alternative explanations. Specifically designed experiments will be required to answer this question.

Our data provide an estimate for the degradation rate of end-plate ACh receptors corrected for non-degradative loss of I-αBT. The rate which we have obtained for the degradation of I-αBT bound to organ-cultured muscles is very close to that found for the loss of I-αBT from end-plate regions of muscle measured by other techniques, when the total rate of loss in the latter experiments is corrected by our estimate of the non-degradative loss of bound I-αBT. Denervation increases the rate of degradation of pre-existing junctional receptors, but the over-all degradation rate for these receptors never reaches that for extrajunctional receptors on denervated muscle. The effect of denervation on the metabolism of pre-existing end-plate receptors is indistinguishable *in vivo* and *in vitro*. Organ-cultured muscle, therefore, is a reliable system for studying metabolism of junctional receptors when the proper procedures are followed. Our observation that the measured degradation rate for receptors in organ-cultured muscle depends on the time between labelling of receptors and the start of organ culture provides an explanation for some of the variability in previous estimates of the degradation rate for end-plate receptors.

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