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N,O-di and *N,N,O*-tri [^{3}H] acetyl α-bungarotoxins as specific labelling agents of cholinergic receptors

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Summary

1. α-Bungarotoxin isolated from the venom of *Bungarus multicinctus* was acetylated with [^{3}H] acetic anhydride and *N*-[^{3}H] acetyl imidazole. Tri-*N*-acetyl and hexa-*N*-acetyl derivatives were obtained from the former, and *N,O*-di, *N,N,O*-tri and *N,N,N,O*-tetraacetyl derivatives from the latter reaction, respectively.

2. There were parallel decreases in both neuromuscular blocking action in the phrenic nerve–diaphragm preparation of rats and depression of acetylcholine response of the rectus abdominis muscle of frogs with increased acetylation. Also, a parallel but greater decrease of toxicity in mice was found.

3. *N,O*-Di and *N,N,O*-triacetyl toxins were localized mostly in the motor endplate region of the rat diaphragm, whereas a slight nonspecific binding along the whole muscle fibre in addition to the peak in the endplate region was observed with *N,N,N,O*-tetraacetyl and tri-*N*-acetyl toxins. In contrast, there was a marked nonspecific binding with hexa-*N*-acetyl toxin and no peak was observed at the endplate zone.

4. The specific binding was saturable and irreversible. The number of toxin-receptive sites in one endplate was $1{\cdot}9–2{\cdot}2 \times 10^7$ for all of the labelled toxins irrespective of their potency.

5. (+)-Tubocurarine protected effectively against the binding as well as the irreversible neuromuscular blocking effect of the toxins.

6. Denervation of the rat diaphragm caused an increase of toxin-receptive sites beginning from the endplate zone at 1–2 days and then along the whole muscle fibre, reaching the maximum at about 18 days. The total receptive sites increased by about 30-fold.

7. The significance of the findings is discussed and it is concluded that *N,O*-di and *N,N,O*-tri-[^{3}H] acetyl α-bungarotoxins are specific and irreversible labelling agents for the cholinergic receptors of skeletal muscle.

Introduction

The specific and irreversible blocking effect on the cholinergic receptor of the motor endplate of α-bungarotoxin (α-BuTX) isolated from the venom of *Bungarus multicinctus* (Chang & Lee, 1963) has recently prompted several groups of authors to use this toxin as a specific probe for the characterization of cholinergic receptors in skeletal muscle (Lee, Tseng & Chiu, 1967 ; Miledi & Potter, 1971 ; Barnard, Wieckowski & Chiu, 1971 ; Berg, Kelly, Sargent, Williamson & Hall, 1972) and in

the electric organ of *Electrophorus* and *Torpedo* (Changeux, Kasai & Lee, 1970; Miledi, Molinoff & Potter, 1971). Preparations of α-BuTX, either iodinated with ^{131}I (Lee & Tseng, 1966; Lee *et al.*, 1967; Miledi & Potter, 1971; Miledi *et al.*, 1971) or ^{125}I (Berg *et al.*, 1972), or acetylated with [^{3}H] acetic anhydride (Barnard *et al.*, 1971) have been used for this purpose. However, these labelled toxins were used without purification, and the question arises whether they were contaminated with the unchanged toxin, or with inactive derivatives, and if so, whether the labelled preparation still possesses the same biological activity as the original toxin. Inclusion of inactive derivatives would also lead to an error in the estimate of binding sites. In this paper, the biological effects and binding properties of five acetyl derivatives of α-BuTX obtained by acetylation with either [^{3}H] acetic anhydride or *N*-[^{3}H] acetyl imidazole were studied, and *N,O*-di and *N,N,O*-tri [^{3}H] acetyl α-BuTX were shown to be stable, specific and irreversible labelling agents of cholinergic receptors at the motor endplate.

Methods

Isolation of α-bungarotoxin

The crude lyophilized venom of *Bungarus multicinctus* was first fractionated into 8 fractions by means of column chromatography on CM-Sephadex C-50 with ammonium acetate buffer gradients, from 0·05 M, pH 5·0 to 1·0 M, pH 6·8, and the largest fraction which contained α-BuTX (Lee, Chang, Kau & Luh, 1972b) was further fractionated by means of CM-cellulose column chromatography with 0·05–0·3 M ammonium acetate buffer as described by Lee *et al.* (1972b) and Mebs, Narita, Iwanaga, Samejima & Lee (1972). The toxin thus isolated appeared as a single band on microzone electrophoresis on cellulose acetate film stained with ponceau-S.

N-[^{3}*H*] *Acetyl imidazole*

The method described by Reddy, Mandell & Goldstein (1963) for acetylation of imidazole was followed. To 25 mg (100 mCi) [^{3}H] acetic anhydride (400 mCi/mmol, New England Nuclear) in 0·1 ml benzene, 18 mg imidazole in 0·2 ml benzene was added at 24° C. After 2 h, the generated acetic acid and benzene were removed under vacuum. The yield was determined by the appearance of absorbancy at 245 nm and found to be quantitative. The specific activity of the formed *N*-[^{3}H] acetyl imidazole was 200 mCi/mmol.

N-[^{3}*H*] *Acetylation*

α-BuTX was *N*-acetylated with [^{3}H] acetic anhydride (400 mCi/mmol, New England Nuclear) according to the procedure described by Fraenkel-Conrat (1957). To 10 mg α-BuTX, dissolved in 0·5 ml 25% (w/v) sodium acetate, was added 12 mg (50 mCi) [^{3}H] acetic anhydride at 0–2° C. Acetylation was stopped at 10 min or 40 min by diluting the reaction mixture with 5 ml cold H_2O; it was then dialyzed in a cellophane bag against 100 ml H_2O in a refrigerator. Water for dialysis was changed every 1 h for 16 hours. The contamination of [^{3}H] acetic acid in [^{3}H] acetyl α-BuTX was estimated from the dialysis curve to be less than 0·2%. The [^{3}H] acetylated α-BuTXs were then freeze-dried.

N,O-[^3H] *Acetylation*

N-Acetyl imidazole preferentially acetylates tyrosine hydroxyl groups although it also reacts with ε- or *N*-terminal free amino groups (Riordan, Wacker & Vallee, 1965 ; Simpson, Riordan & Vallee, 1963). To 18 mg *N*-[^{3}H] acetyl imidazole (200 mCi/mmol) dissolved in 2·5 ml 0·01 M tris buffer was added 20 mg α-BuTX at 25° C. The pH was readjusted to 7·5 and the reaction was allowed to continue for 2 hours. The reaction mixture was then dialyzed against 400 ml cold H_2O for 12 h with 8 changes of water. After lyophilization the mixture of acetylated products was purified by means of starch zone electrophoresis as described for α-BuTX (Chang & Lee, 1963). An average potential of 5 V/cm was applied at 0–4° C for 24 hours. Aqueous extracts (7 ml) of each 1 cm starch segment were assayed for protein content by optical absorbancy at 278 nm and for radioactivity by liquid scintillation counting. The recovery of protein was about 80%. Fractions belonging to the same peak were then pooled, dialyzed against 400 ml cold H_2O for 8 h (changing the dialyzing water every 1–1·5 h) and lyophilized. The contamination of [^{3}H] acetic acid, estimated from its dialysis curve, was less than 0·2%.

Determination of labile O-[^3H] *acetyl group*

The procedure for deacetylation of tyrosine *O*-acetyl group by hydroxylamine (Simpson *et al.*, 1963) was followed. To 0·5 ml of a solution of *O*-[^{3}H] acetyl α-BuTX containing 60–85 μg protein was added 0·15 ml 4 N hydroxylamine and the pH was adjusted to 6·5. After 2 h at 25° C, the liberated [^{3}H] acetic acid was determined by equilibrium dialysis against 20 ml H_2O for 48 h in a refrigerator. Control experiments showed that all the [^{3}H] acetylated α-BuTXs were stable under these conditions when no hydroxylamine was added.

Toxicity in mice

The toxicity of each fraction was determined by intraperitoneal injection in mice (NIH strain, 15–20 g in weight). Because of the limited amount of labelled toxins, 5 mice were used for each of three doses. The median lethal dose was calculated graphically according to the method of Litchfield & Wilcoxon (1949).

Neuromuscular blocking action

The blocking effect on neuromuscular transmission was determined in the phrenic nerve–diaphragm preparation of Long Evans rats of body weight 220–300 g. Two hemidiaphragms were set up in an organ bath containing 30 ml Tyrode solution oxygenated with 95% O_2+5% CO_2 at 37° C unless otherwise mentioned. The phrenic nerve was stimulated with a single supramaximal rectangular pulse every 10 seconds. The relative potency was obtained by measuring the time needed to cause complete paralysis by each toxin and comparing this with a calibration curve (Fig. 1) of blocking time against concentration of unmodified α-BuTX. Isometric recording with a force-displacement transducer was used. In another experiment, the contraction of the rectus abdominis muscle of *Rana tigrina* in response to acetylcholine ($1{\cdot}1\times10^{-5}$M) was studied. The organ bath contained 10 ml frog Ringer solution at 22–25° C.

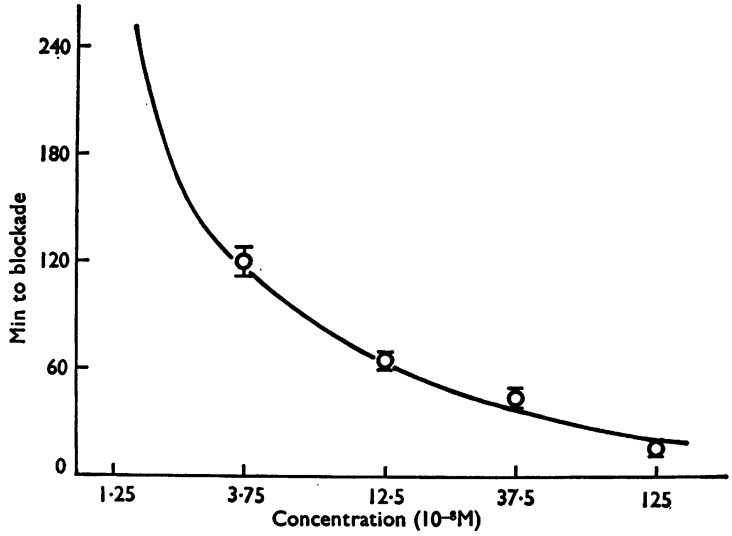

FIG. 1. Times needed for the complete neuromuscular blockade of the rat phrenic nerve–diaphragm preparation at various concentrations of α-bungarotoxin at 37° C. Vertical bars indicate S.E. of 3–7 experiments.

Binding studies

The binding of the labelled toxins was studied in the rat diaphragm. The muscle was trimmed to about 70% of its original width and incubated with the toxin for 2 h under the conditions described above for the assay of the neuromuscular blocking effect. It was then washed repeatedly with 50 ml Tyrode solution for 5 h with 8–12 changes of bathing solution. The treated diaphragms were then stretched on paper, dried and cut into segments 1·5 mm wide, parallel to the endplate zone. Each segment was digested by adding 0·2 ml 0·5 N KOH at 50° C and the radioactivity measured by a Packard liquid scintillation spectrometer with 10 ml phosphor solution consisting of toluene 7 ml, ethyleneglycol monomethylether 3 ml, PPO 20 mg, POPOP 0·5 mg, naphthalene 300 mg. Quenching was corrected for by the external standard method.

Results

N,O-[^3H] *Acetylation*

Under the experimental conditions given in the **Methods**, several [^{3}H] acetyl groups were incorporated in the molecule of α-BuTX. On starch zone electrophoresis the reaction product appeared to be a mixture composed of at least four components, none of which was unchanged α-BuTX (Fig. 2). All of the derivatives were less mobile than the original toxin. There were also four corresponding peaks of radioactivity. The [^{3}H] acetyl groups incorporated per molecule of the derivatives were found to be 2·04, 3·13, 3·64 and 1·65 respectively for the peaks I, II, III and IV in the order of decreasing mobility. Being far from integral numbers, values of 3·64 and 1·65 might indicate incomplete separation of fraction III and IV. It appears that the more acetyl groups are incorporated the more electromobility

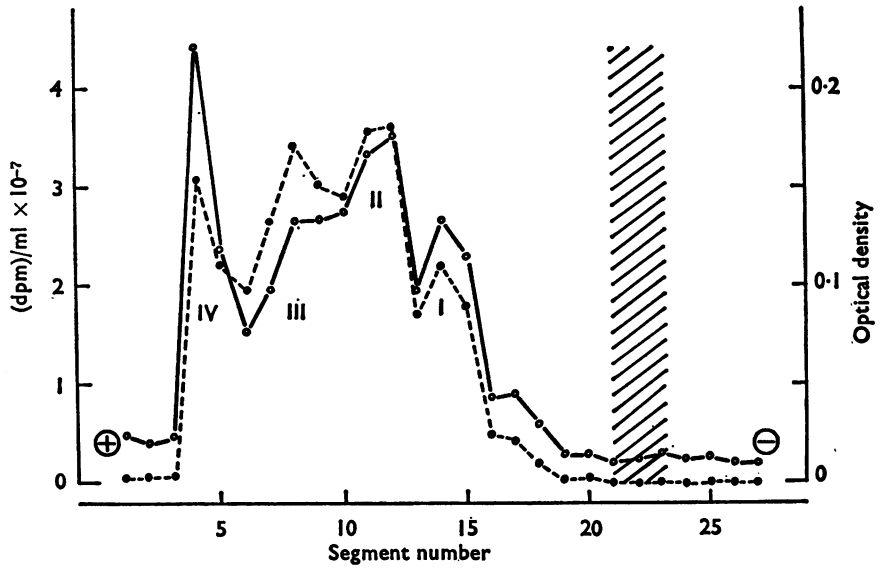

FIG. 2. The pattern of starch zone electrophoresis of α-bungarotoxin acetylated with *N*-[³H] acetyl imidazole. The reaction product was applied at segment No. 4. Optical density at 278 nm (○——○) and radioactivity (●- - - -●) of 7 ml extracts of each 1 cm segment are shown. Shaded area is included to show the migration of unmodified α-BuTX under the same conditions.

decreases except for the least movable fraction (IV). Since the latter fraction was found to have neither toxicity nor curare-like action, it will not be considered further.

Deacetylation of the labile tyrosine *O*-acetyl group by hydroxylamine showed that there were 0·97, 0·65 and 0·68 *O*-[³H] acetyl groups in each molecule of fraction I, II and III, respectively. This result indicates that these three derivatives probably contained one *O*-acetyl group in their molecules although there are two tyrosine residues in the toxin molecule (Mebs, *et al.*, 1972). Therefore, the other [³H] acetyl groups must be on amino groups. This view is further supported by the proportional decrease of mobility on electrophoresis as the incorporation of acetyl group increased (Fig. 2). These derivatives will be henceforth designated as *N,O*-di, *N,N,O*-tri and *N,N,N,O*-tetraacetyl α-BuTX, respectively.

N-[³*H*] *Acetylation*

When the acetylation with [³H] acetic anhydride was allowed to proceed for only 10 min, the reaction product was found to have 3·07 [³H] acetyl groups in one molecule of α-BuTX. Microzone electrophoresis revealed that no unmodified α-BuTX was present and the product was composed of two derivatives. At pH 7·8 both acetylated toxins migrated towards the anode, while the original α-BuTX migrated to the cathode, indicating a change in isoelectric point due to acetylation of free amino groups. No attempt was made to separate the *N*-acetylated derivatives.

When acetylation was continued for 40 min, the product contained 6·0 [³H] acetyl groups per molecule of α-BuTX. It has previously been shown that six free amino

groups in the original toxin disappear under comparable conditions of acetylation (Chang, 1970). Thus acetylation takes place exclusively at free amino groups under these conditions (Fraenkel-Conrat, 1957). The products of acetylation for 10 and 40 min will be henceforth designated as tri-*N*-acetyl and hexa-*N*-acetyl α-BuTX, respectively.

Neuromuscular blocking action

The neuromuscular blocking activity of each labelled acetyl α-BuTX was assessed from the time needed for the compound at a concentration of $1{\cdot}25\times10^{-7}$M to cause a complete blockade in the indirectly stimulated rat phrenic nerve-diaphragm preparation. The relative potencies are illustrated in Table 1. *N,O,*-Diacetyl α-BuTX was the most potent and possessed about 80% of the original activity. The activity further decreased as more acetyl groups were incorporated. The data in Table 1 indicate that *N*-acetylation affected the neuromusclar blocking activity to a greater extent than *O*-acetylation. Hexa-*N*-acetyl toxin was the least active, but still had about 20% of the original activity. This result shows that the amino group may not be essential for the biological action as suggested by Karlsson & Eaker (1972) for the neurotoxin isolated from *Naja naja siamensis*. The blocking effects of all of the acetylated toxins, like the original one, were confined to the neuromuscular junction, leaving the contraction in response to direct stimulation unaffected. No recovery was observed after repeated washing for up to 5 hours.

Toxicity

The toxicity in mice by intraperitoneal injection of each fraction is also shown in Table 1. The decrease in toxicity generally paralleled the decrease in neuromuscular blocking activity. However, the toxicity decreased more than the neuromuscular blocking activity, indicating that some changes may have occurred in the absorption, metabolism, nonspecific binding or excretion in addition to the change of neuromuscular blocking activity in the modified molecules.

Specificity of binding to acetylcholine receptors

The specificity of binding to acetylcholine receptors at the motor endplate was determined by measuring the distribution of radioactivity in the diaphragm muscle. The preparation was incubated with each of the labelled derivatives at a concentration of $1{\cdot}25\times10^{-7}$M, for 2 h at 37° C and then washed with plain Tyrode

TABLE 1. *Toxicity in mice, neuromuscular blocking action in rat diaphragm and depression of acetylcholine response in frog rectus abdominis muscle of acetylated bungarotoxins (α-BuTX). The relative potency of each labelled toxin to the original α-BuTX was obtained as described in* **Methods**

Toxins	Toxicity LD50 (μg/g)	Toxicity per cent	Neuromuscular blocking action (Mean±S.E.)	Depression of ACh response (n=2)
α-BuTX	0·16	100	100	100
N,O-Diacetyl α-BuTX	0·42	38	79±22·5 (n=3)	54–55
N,N,O-Triacetyl α-BuTX	0·46	35	56± 9·2 (n=4)	50–73
N,N,N,O-Tetraacetyl α-BuTX	0·46	35	47± 5·8 (n=4)	17–20
Tri-*N*-acetyl α-BuTX	0·54	29	31± 3·3 (n=4)	18–21
Hexa-*N*-acetyl α-BuTX	1·55	10	20, 24 (n=2)	9–11

solution for 5 hours. As illustrated in Fig. 3, when *N,O*-diacetyl α-BuTX or *N,N,O*-triacetyl α-BuTX was used, most of the radioactivity was concentrated in the central 2 strips containing the motor endplates whereas the other part of the muscle bound only negligible amounts of toxin. On the other hand, a slight degree of nonspecific binding along the whole muscle fibre in addition to the specific binding at the endplate zone was observed with *N,N,N,O*-tetraacetyl and tri-*N*-acetyl α-BuTX (Fig. 3). In contrast, hexa-*N*-acetyl toxin gave no peak at the endplate zone and there was marked radioactivity along the whole muscle fibre even though its pharmacological action was confined to the neuromuscular junction.

Saturation of binding

The binding of *N,O*-diacetyl α-BuTX was studied with various concentrations of the toxin. The relation between the concentration and the binding at the endplate zone obtained after 2 h incubation is shown in Figure 4. The binding was almost maximum at a concentration of $3{\cdot}75\times10^{-8}$M, which was just enough to cause a complete neuromuscular blockade in 2 h, and no further increase in binding could be obtained by increasing the concentration from $1{\cdot}25\times10^{-7}$ to $1{\cdot}25\times10^{-6}$M. It is thus evident that the binding of labelled toxin at the motor endplate is saturable. Similar results were found for the binding of *N,N,O*-triacetyl α-BuTX. In the case of tri-*N*-acetyl and *N,N,N,O*-tetraacetyl α-BuTX, both the neuromuscular blockade and saturation of binding needed a concentration higher than $1{\cdot}25\times10^{-7}$M. The complete blockade of neuromuscular transmission appeared to be associated with more than 80% occupation of the sites for all of the labelled toxins irrespective of their potency.

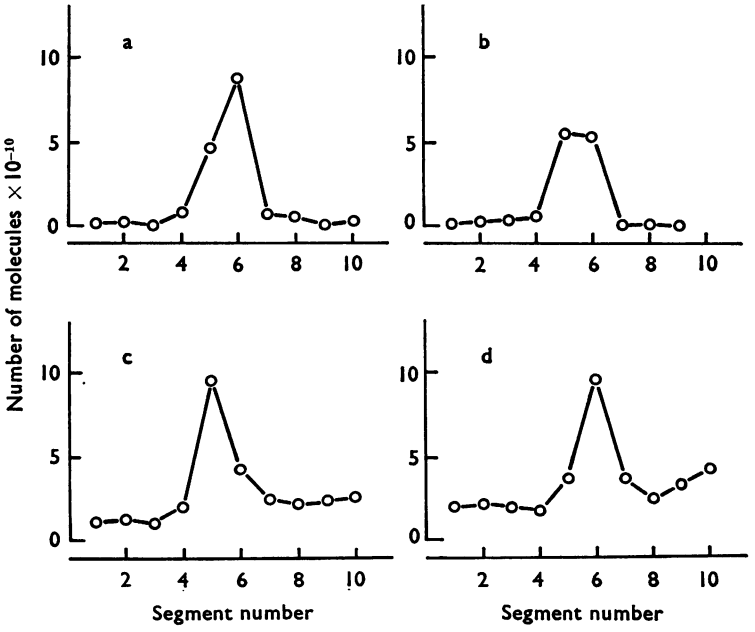

FIG. 3. Distribution of four acetylated labelled α-bungarotoxins in the rat diaphragm. The diaphragm was incubated with each of the toxins for 2 h at a concentration of $1{\cdot}25\times10^{-7}$M and washed for 5 hours. All segments represent 1·5 mm width of diaphragm and are numbered from the central tendon (abscissae). The number of toxin molecules in each segment is shown on the ordinates. a: *N,O*-diacetyl, b: *N,N,O*-triacetyl, c: *N,N,N,O*-tetraacetyl, and d: tri-*N*-acetyl α-BuTX.

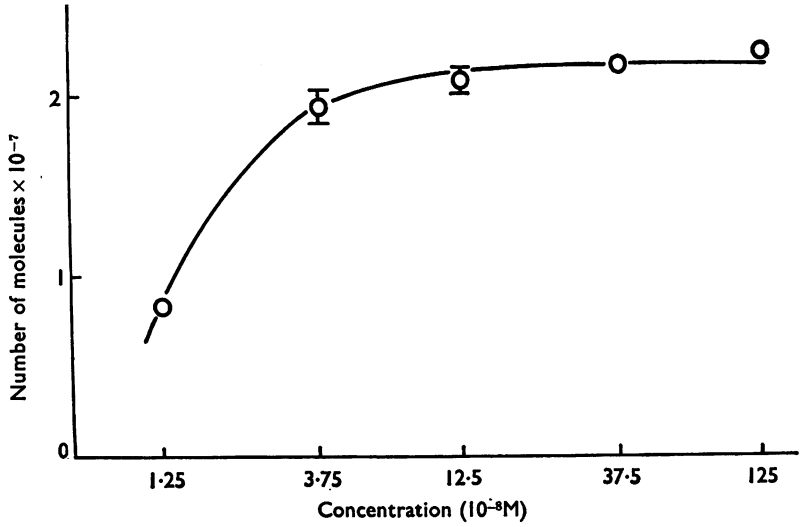

FIG. 4. The number of *N,O*-diacetyl α-bungarotoxin molecules bound with each endplate of the rat diaphragm at various concentrations (n=2–4). The duration of incubation was 2 hours. Vertical bars indicate the S.E. of 4 experiments.

The number of molecules of each modified toxin bound to each endplate of the rat diaphragm was calculated from the value obtained at saturation by assuming that the isolated diaphragm contained 70% of the total of 10,000 muscle fibres as estimated by Krnjević & Miledi (1958), with one endplate per fibre. The numbers appear to be close to each other for all of the four labelled toxins (Table 2) and are in the range $1{\cdot}93$–$2{\cdot}16 \times 10^7$ molecules per endplate.

Irreversibility of specific binding

The distribution of *N,O*-diacetyl α-BuTX was studied after 30 min, 3 and 5 h of washing. There was pronounced nonspecific binding over the whole muscle fibre after washing for 30 min but it was mostly removed after washing for 3 hours (Fig. 5). The specific binding at the endplate zone, however, was the same, $2{\cdot}0$–$2{\cdot}2 \times 10^7$ molecules per endplate whether the muscle was washed for 30 min, 3 h or 5 hours (Fig. 5).

Prevention of binding by (+)-tubocurarine

The rat phrenic nerve-diaphragm preparation was incubated with *N,O*-diacetyl α-BuTX ($1{\cdot}25 \times 10^{-7}$M) for 2 h in the presence of (+)-tubocurarine, $1{\cdot}27 \times 10^{-4}$M

TABLE 2. *The number of toxin-receptive sites in the motor endplate region of the rat diaphragm. The muscle was incubated with each of the toxins at 37° C for 2 h at concentrations which saturate the receptive sites*

Toxins	Concentration (10^{-7} M)	Number of molecules per endplate (Means±S.E., $\times 10^7$)
N,O-Diacetyl α-BuTX	1·25	2·07±0·06 (n=4)
N,N,O-Triacetyl α-BuTX	1·25	1·93±0·03 (n=3)
N,N,N,O-Tetraacetyl α-BuTX	3·75	2·16±0·30 (n=4)
Tri-*N*-acetyl α-BuTX	3·75	1·96±0·16 (n=4)

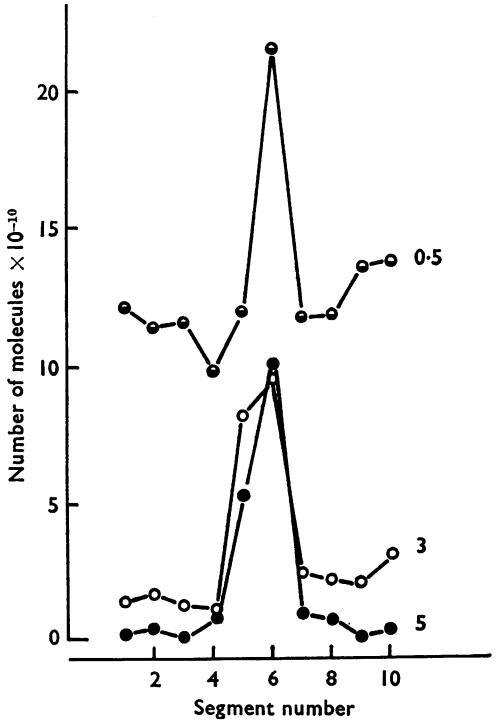

FIG. 5. The distribution of *N,O*-diacetyl α-bungarotoxin in the rat diaphragm after various periods of repeated washings. The diaphragm was incubated with $1{\cdot}25\times10^{-7}$M toxin for 2 hours. Numerals in the figure denote the period (h) of washing. The ordinates show the number of toxin molecules in each segment. Note that the specific binding in the endplate region was not reduced by washing.

at 37° C and then washed, first with Tyrode solution containing $1{\cdot}27\times10^{-5}$M (+)-tubocurarine for 1 h, and then with plain Tyrode solution for 3 hours. The contraction in response to indirect stimulation recovered to 10–30% of its control amplitude and the binding at the endplate zone was reduced by 48% (Table 3). A similar experiment with *N,N,O*-triacetyl toxin gave 65% protection from binding. When incubation with *N,O*-diacetyl α-BuTX was carried out at 32° C a greater degree of protection was observed (Table 3). An almost complete protection against binding and recovery of twitch response was achieved with the same concentration of (+)-tubocurarine when a lower concentration of *N,O*-diacetyl α-BuTX ($3{\cdot}75\times10^{-8}$M, which still bound to most of the receptive site in 2 h), was used (Table 3).

TABLE 3. *Prevention by (+)-tubocurarine of the binding of acetylated α-bungarotoxins (α-BuTX) to the motor endplate of the rat diaphragm. (+)-Tubocurarine,* $1{\cdot}27\times10^{-4}$ M, *was present during the 2-h period of incubation with toxins and* $1{\cdot}27\times10^{-5}$ M, *during the first hour of washing*

Toxins	Concentration ($\times10^{-8}$ M)	Temperature	Molecules of toxin bound: Without (+)-tubocurarine ($\times10^7$)	Molecules of toxin bound: With (+)-tubocurarine ($\times10^7$)	% Inhibition of binding
N,O-Diacetyl α-BuTX	12·5	37°	2·07±0·06 (*n*=4)	1·10±0·16 (*n*=4)	48
N,O-Diacetyl α-BuTX	3·75	37°	1·92±0·11 (*n*=4)	0·25±0·48 (*n*=4)	87
N,O-Diacetyl α-BuTX	12·5	32°	1·97, 2·11 (*n*=2)	0·65, 0·66 (*n*=2)	67
N,N,O-Triacetyl α-BuTX	12·5	37°	1·93±0·03 (*n*=3)	0·66, 0·70 (*n*=2)	65

Binding in the denervated muscle

To study further the specificity of the binding of labelled toxin with the acetylcholine receptor, the increase and spread of the receptor sites for *N,O*-diacetyl α-BuTX were studied at various intervals after section of the left phrenic nerve in the neck. The indirect twitch response was depressed by about 70% 24 h after nerve section and complete blockade occurred at 48 hours. Miledi & Slater (1970), however, found that the endplate potential disappeared in most of the superficial muscle fibre within 24 hours. As illustrated in Fig. 6, the number of binding sites at the endplate region started to increase 24–48 h after denervation when the degeneration of the nerve was complete. After 4 days there was a marked increase along the whole length of the muscle fibres as well as at the endplate zone. The increase in binding sites continued further up to 18 days after denervation. In agreement with the autoradiographic finding of Lee *et al.* (1967) the peak in the endplate region was still visible in all of the denervated muscle. The total number of binding sites increased about 30 fold 18 days after denervation. This result confirmed that obtained by Miledi & Potter (1971). The time-course of spread of binding sites corresponds with the spread of acetylcholine-sensitive sites after denervation reported by Axelsson & Thesleff (1959). It is of interest to note that the specific binding in the contralateral innervated diaphragm also increased slightly to $2{\cdot}6$–$2{\cdot}8 \times 10^7$ molecules per endplate.

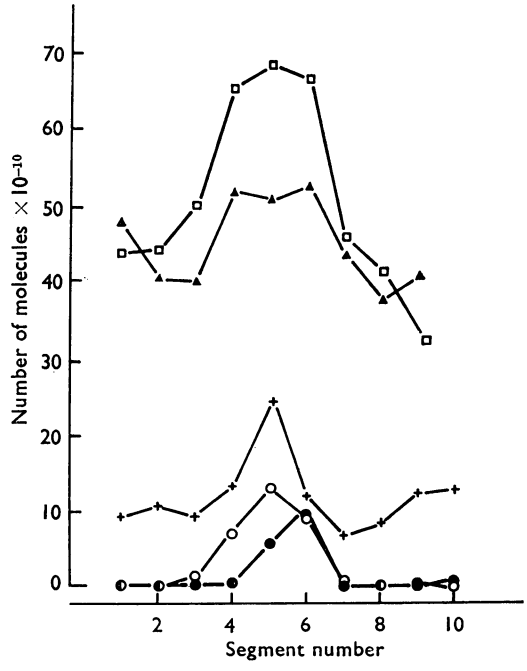

FIG. 6. The distribution of *N,O*-diacetyl α-bungarotoxin in the diaphragm denervated for 2–18 days. The diaphragm was isolated after denervation and incubated with the toxin, $1{\cdot}25 \times 10^{-7}$M for 2 h at 32° C and then repeatedly washed for 5 hours. The ordinates represent the number of toxin molecules in each segment. ●——●, Control; ○——○, 2; +——+, 4 ▲——▲, 8; □——□, 18; 8 days after phrenicotomy.

Effect on frog rectus abdominis

Recent experiments (Lee, Chang & Chen, 1972a) reveal that the reversibility of the neuromuscular blocking action of neurotoxins of elapid venoms varies with their amino acid composition as well as the species of nerve-muscle preparation studied. Among these neurotoxins, α-BuTX is unique in that it acts irreversibly in all of the muscles so far tested. On the other hand, the sartorius muscle of *Rana tigrina* is also unique in that it is affected reversibly by all of the elapid neurotoxins except α-BuTX. The effects of the labelled α-BuTXs were therefore studied on the response to acetylcholine of the rectus abdominis muscle of this particular species. It was found that the potency of these derivatives in antagonizing the acetylcholine response varied in parallel with their neuromuscular blocking activities in the rat diaphragm (Table 1). However, upon washing the response of the rectus abdominis gradually recovered (Fig. 7).

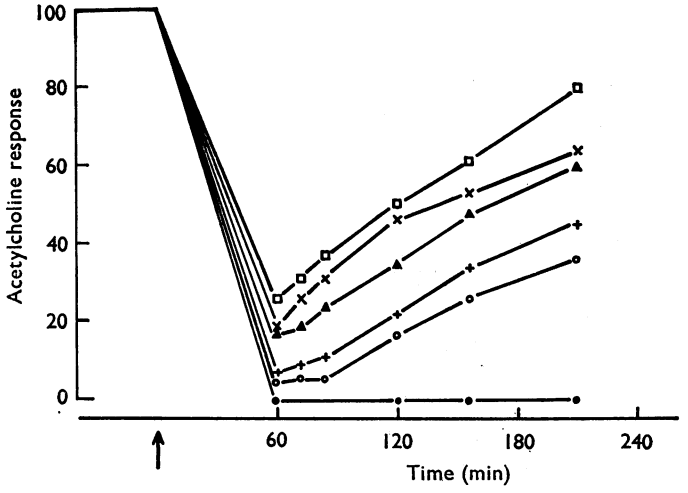

FIG. 7. Effects of acetylated α-bungarotoxins on the acetylcholine response of rectus abdominis muscle of *Rana tigrina* and the recovery of response after washings. The muscle was incubated with $1{\cdot}25\times10^{-7}$M α-BuTX (●——●), *N,O*-diacetyl (+——+), *N,N,O*-triacetyl (○——○), *N,N,N,O*-tetraacetyl (×——×), tri-*N*-acetyl (▲——▲) or hexa-*N*-acetyl α-BuTX (□——□) for 60 min and then washed. The response to acetylcholine is expressed as % of controls before treatment with each toxin.

Discussion

The specificity and irreversibility of the pharmacological action of α-BuTX on the acetylcholine receptor in the motor endplate are well established (Chang & Lee, 1963). The parallel decreases of toxicity in mice, neuromuscular blocking action in the rat diaphragm and depression of the acetylcholine response of frog rectus abdominis muscle by the labelled toxins indicate that, like the unmodified α-BuTX, the postsynaptic neuromuscular blocking action is the main action of these labelled derivatives. These toxins acetylated on amino and tyrosine hydroxyl groups also appear to have the characteristic mode of action at the receptors of the motor endplate. The close relationship between the site of acetylcholine sensitivity and the localization of acetylated α-BuTXs and the prevention of binding of these labelled toxins by (+)-tubocurarine strongly suggest that the bind-

ing site of these toxins is at least very closely related to or even identical with that of acetylcholine.

The finding that the time-course of the appearance and spread of the toxin-receptive site after denervation corresponds to that of acetylcholine-sensitive sites (Axelsson & Thesleff, 1959), also points to the close relationship between the toxin-receptive site and the newly generated acetylcholine receptor. In this respect, the binding of the acetylated toxins is even more specific than that of (+)-tubocurarine since the binding of (+)-tubocurarine does not appear to be increased in denervated muscle (Waser, 1967). The irreversibility of the action of α-BuTX is retained in the acetylated derivatives as revealed by their persistent neuromuscular blocking effect in the rat diaphragm and their unreduced binding at the motor endplate region upon repeated washing. In the rectus abdominis of *Rana tigrina*, however, acetylated toxins acted reversibly, indicating some difference in the binding in this particular species. Among the five acetylated toxins, *N,O*-di and *N,N,O*-triacetyl αBuTXs appear to be more active than the others and suited for the study of cholinergic receptors. These two toxins also have less nonspecific binding than others.

The labelled preparations obtained by Lee *et al.* (1967), Miledi & Potter (1971), Barnard *et al.* (1971) and Berg *et al.* (1972) also appear to act similarly to the original α-BuTX. However, the composition of these preparations seems to be complex. While no data are available for the calculation of the fraction of ^{131}I label for the preparations made by Lee *et al.* (1967) and Miledi & Potter (1971), the preparation of Berg *et al.* (1972) contained only 0·05 mol of ^{125}I label and that of Barnard *et al.* (1971) contained 0·4–0·8 mol of [^{3}H] acetyl label per mol of toxin. Bosmann (1972) also claimed to have prepared [^{3}H] acetyl α-BuTX with [^{3}H] acetic anhydride. However, calculations from his data show that there would be 30 acetyl groups per molecule of toxin, although there are only six lysine and total of 74 amino acid residues in the molecule of α-BuTX (Mebs *et al.*, 1972). Surprisingly, the isoelectric point was not changed, indicating that the lysine residues were not modified. It is possible, therefore, that no acetylation actually occurred in Bosmann's preparation since, under the conditions employed, acetylation takes place preferentially on the free amino group (Fraenkel-Conrat, 1957). The radio-activity of his preparation might have come from incomplete desalting. Labelled preparations of other neurotoxins isolated from cobra venoms such as α-neurotoxin from *Naja nigricollis* (Menez, Morgat, Fromageot, Ronseray, Boquet & Changeux, 1971) and neurotoxin from *Naja naja siamensis* (Cooper, Smith & Reich, 1971) have also been used as labelling agents for acetylcholine receptors. However, these neurotoxins act reversibly in some muscles, such as the sartorius muscle of *Rana tigrina* and the diaphragm of cats (Lee *et al.*, 1972a).

The number of toxin-binding sites per endplate of the rat diaphragm was in the close range $1{\cdot}9–2{\cdot}2 \times 10^7$, no matter which acetylated toxin was used. It is likely therefore that the variation in the potency as neuromuscular blocking agents among these acetyl derivatives is due to difference in the rate of binding, and that the degree of neuromuscular blockade is dependent on the proportion of receptive sites occupied by the toxins. The number of sites obtained in the present experiment is close to that obtained by Miledi & Potter (1971), $4{\cdot}7 \times 10^7$, and Berg *et al.* (1972), $2{\cdot}9 \times 10^7$ (calculated by assuming that a hemidiaphragm weighs 200 mg), in the rat diaphragm and that by Barnard *et al.* (1971), 3×10^7, in the mouse diaphragm. It

is also similar to the number of catalytic sites of cholinesterase at the motor endplate of mouse diaphragm (Waser, 1967; Barnard & Rogers, 1967). Whether the small difference in the number of toxin-receptive sites between ours and others is meaningful is not known. It is likely, however, that if there were inactive impurities contained in the unpurified labelled preparations, then the calculated number of receptive sites would be higher than it should be.

In the experiment of Miledi & Potter (1971) with the sartorius of frogs, the maximum inhibition of the binding of the iodinated toxin by (+)-tubocurarine was 50%. We have found, however, that when the concentration of *N,O*-diacetyl α-BuTX was reduced, the protection could be as high as 87% in the rat diaphragm. Thus, it is likely that the toxin-receptive sites in the motor endplate are homogeneous rather than heterogeneous as previously suggested (Miledi & Potter, 1971). The reason for the small number of molecules of (+)-tubocurarine (4×10^6) bound to an endplate at complete neuromuscular blockade (Waser, 1967) in comparison with the number of α-BuTX-receptive sites in our experiment and in others is not known. It may be due to a wash-out of (+)-tubocurarine, as proposed by Barnard *et al.* (1971), or to an allosteric nature of the action, as proposed by Waser (1967) and Miledi & Potter (1971).

The slight increase of toxin-binding sites in the endplate region of the innervated diaphragm of rats whose contralateral diaphragm was denervated is of interest. Since denervation of one hemidiaphragm may increase the nerve activity in the other, it may be suggested that the number of cholinergic receptors at the endplate is influenced by the rate of nerve impulses or the amount of the transmitter released.

Note added in proof. Recently, Fambrough, D. M. & Hartzell, H. C. (*Science*, **176**, 189–191, 1972) by using purified ^{125}I-α-BuTX, have found the number of receptive sites in the rat diaphragm to be $1{\cdot}40$–$3{\cdot}98 \times 10^7$ per endplate, being nearly proportional to the body weight.

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