Brown, M. F., & Seelig, J. (1978) *Biochemistry 17*, 381–384.
Browning, J. L., & Seelig, J. (1980) *Biochemistry 19*, 1262–1270.
Büldt, G., Gally, H. U., Seelig, A., Seelig, J., & Zaccai, G. (1978) *Nature (London) 271*, 182–184.
Büldt, G., Gally, H. U., Seelig, J., & Zaccai, G. (1979) *J. Mol. Biol. 134*, 673–691.
Chapman, D., Peel, W. E., Kingston, B., & Lilley, T. H. (1977) *Biochim. Biophys. Acta 464*, 260–275.
Davis, J. H., Jeffrey, K. R., Bloom, M., Valic, M. I., & Higgs, T. P. (1976) *Chem. Phys. Lett. 42*, 390–394.
Gally, H. U., Niederberger, W., & Seelig, J. (1975) *Biochemistry 14*, 3647–3652.
Gally, H. U., Pluschke, G., Overath, P., & Seelig, J. (1981) *Biochemistry 20*, 1826–1831.
Grasdalen, H., Eriksson, L. E. G., Westman, J., & Ehrenberg, A. (1977) *Biochim. Biophys. Acta 469*, 151–162.
Hauser, H., & Phillips, M. C. (1979) *Prog. Surf. Membr. Sci. 13*, 297–413.
Hauser, H., Phillips, M. C., Levine, B. A., & Williams, R. J. P. (1975) *Eur. J. Biochem. 58*, 133–144.
Hauser, H., Phillips, M. C., Levine, B. A., & Williams, R. J. P. (1976) *Nature (London) 261*, 390–394.
Hauser, H., Hinckley, C. C., Krebs, J., Levine, B. A., Phillips, M. C., & Williams, R. J. P. (1977) *Biochim. Biophys. Acta 468*, 364–377.
Hauser, H., Guyer, W., Levine, B., Skrabal, P., & Williams, R. J. P. (1978) *Biochim. Biophys. Acta 508*, 450–463.
Hutton, W. C., Yeagle, P. L., & Martin, R. B. (1977) *Chem. Phys. Lipids 19*, 255–265.
Mantsch, H. H., Saito, H., & Smith, I. C. P. (1977) *Prog. Nucl. Magn. Reson. Spectrosc. 11*, 211–272.
McLaughlin, A., Grathwohl, C., & McLaughlin, S. (1978) *Biochim. Biophys. Acta 513*, 338–357.
Robinson, R. A., & Stokes, R. H. (1949) *Trans. Faraday Soc. 45*, 612–624.
Robinson, R. A., & Stokes, R. H. (1959) *Electrolyte Solutions*, 2nd ed., p 305, Butterworth, London.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–418.
Seelig, J. (1978) *Biochim. Biophys. Acta 515*, 105–140.
Seelig, J., & Gally, H. (1976) *Biochemistry 15*, 5199–5204.
Seelig, J., Gally, H., & Wohlgemuth, R. (1977) *Biochim. Biophys. Acta 467*, 109–119.
Seelig, J., Tamm, L., Hymel, L., & Fleischer, S. (1981) *Biochemistry 20*, 3922–3932.
Skarjune, R., & Oldfield, E. (1979) *Biochemistry 18*, 5903–5909.
Spedding, F. H., Weber, H. O., Saeger, V. W., Petheram, H. H., Rard, J. A., & Habenschuss, S. (1976) *J. Chem. Eng. Data 21*, 341–360.
Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., & Smith, I. C. P. (1976) *Biochemistry 15*, 954–966.
Westman, J., & Eriksson, L. E. G. (1979) *Biochim. Biophys. Acta 557*, 62–68.
Wohlgemuth, R., Waespe-Šarčević, N., & Seelig, J. (1980) *Biochemistry 19*, 3315–3321.

Radiolabeled α-Bungarotoxin Derivatives: Kinetic Interaction with Nicotinic Acetylcholine Receptors†

Ronald J. Lukas,‡ Hiromi Morimoto, Michael R. Hanley,§ and Edward L. Bennett*

ABSTRACT: The binding interactions of purified tritiated [^{3}H]-α-Bgt and monoiodinated and diiodinated derivatives of α-bungarotoxin with membrane-bound nicotinic acetylcholine receptors (nAChR) from *Torpedo californica* electroplax and rat brain have been characterized by several kinetic and equilibrium techniques. By all criteria, [^{3}H]-α-Bgt and ^{125}I-labeled monoiodinated α-Bgt ([^{125}I]-α-Bgt) exhibited comparable specificities and affinities for nAChR. In contrast, affinity of nAChR for ^{125}I-labeled diiodinated α-Bgt ([$^{125}I_2$]-α-Bgt) was reduced, and [$^{125}I_2$]-α-Bgt–nAChR complexes showed anomalous biphasic dissociation kinetics. [^{125}I]-α-Bgt and [$^{125}I_2$]-α-Bgt binding was inhibited most potently by native α-Bgt as opposed to iodinated toxins. [^{3}H]-α-Bgt was the radiotoxin most resistant to inhibitory influences. The use of well-characterized, chemically modified α-Bgt derivatives may identify ligand binding microheterogeneities and tissue-specific receptor subclasses.

Utilization of curaremimetic neurotoxins as specific probes for nAChR[1] has contributed to our knowledge of neurotransmitter receptor structure and function (Lee, 1971; Heidmann & Changeux, 1978). Despite the wide diversity of available toxins, and a significant literature on effects of chemical modification on their toxic activity (Tu, 1977), use of different naturally occurring or chemically modified toxins to probe receptor properties has been limited.

In an earlier communication [Lukas(iewicz) et al., 1978], we reported that column-purified native α-Bgt and tritiated, monoiodinated, and diiodinated α-Bgt derivatives exhibit characteristically different ultraviolet and circular dichroism spectra, suggesting that progressive iodination of an exposed tyrosine residue(s) leads to alterations in toxin secondary

† From the Chemical Biodynamics Division, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720. *Received April 14, 1981.* Supported in part by the Director of Energy Research, Office of Life Sciences Research and Nuclear Medicine Applications, Division of General Life Sciences of the U.S. Department of Energy, under Contract W-7405-ENG-48. A preliminary account of these results was presented at the Ninth Annual Meeting of Society for Neuroscience (Bennett et al., 1979).

‡ Permanent address: Division of Neurobiology, Barrow Neurological Institute, Phoenix, AZ 85013.
§ Present address: MRC Neurochemical Pharmacology Unit, Medical School, Cambridge CB2 2QH, England.

[1] Abbreviations used: α-Bgt, α-bungarotoxin; nAChR, nicotinic acetylcholine receptors; monoiodo-α-Bgt, monoiodinated α-Bgt; diiodo-α-Bgt, diiodinated α-Bgt; [^{3}H]-α-Bgt, tritium-labeled α-Bgt; [^{125}I]-α-Bgt, ^{125}I-labeled monoiodinated α-Bgt; [$^{125}I_2$]-α-Bgt, ^{125}I-labeled diiodinated α-Bgt.

0006-2960/81/0420-7373$01.25/0

structure. Radiotoxin binding competition data further suggested that iodination also leads to some disruption of receptor recognition properties, presumably as a consequence of structural alterations.

In order to quantitatively document any such alterations in receptor binding characteristics, we examined kinetic properties of the reaction of [^{3}H]-α-Bgt, [^{125}I]-α-Bgt, and [$^{125}I_2$]-α-Bgt derivatives with both membrane-bound nAChR from *Torpedo californica* electric organ and rat brain membrane fractions.

Experimental Procedures

Materials. Crude venom from *Bungarus multicinctus* (Miami Serpentarium, Miami, FL), *d*-tubocurarine chloride (Calbiochem), and carbamylcholine chloride (Sigma Chemical Co.) were stored at −20 °C. Wag/Rig rats were from the Lawrence Berkeley Laboratory rat colony. Liquid nitrogen frozen chunks of *T. californica* electric organ were obtained from Pacific Biomarine, Ventura, CA, and stored at −80 °C.

Preparation of α-Bgt followed procedures of Eterović et al. (1975b), as modified by Lukas(iewicz) et al. (1978). Iodination of α-Bgt and resolution of monolabeled, dilabeled, and unlabeled toxins via ion-exchange column chromatography at pH 6.5 were carried out as described previously [Lukas(iewicz) et al., 1978]. Tritiated α-Bgt was prepared according to Eterović et al. (1975a) using purified monoiodo-α-Bgt and resolved from residual iodinated species by ion-exchange chromatography at pH 6.5 [Lukas(iewicz) et al., 1978]. Nonradiolabeled α-Bgt (25 μM) fractions were stored in 1-mL aliquots at −20 °C until use and kept at 0 °C thereafter. Radiolabeled α-Bgt derivatives (10–25 μM) were stored at −20 °C in the presence of 1 mg/mL bovine serum albumin.

Toxin and Radiotoxin Concentrations, Radiopurity, and Specific Activities. Concentrations were determined from optical absorbance measurements (Cary 118 spectrophotometer) at 280 nm, corrected for scattering, using $\epsilon_{280}^{0.1\%} = 1.32$ for the native, tritiated, and monoiodinated α-Bgt and $\epsilon_{280}^{0.1\%} = 1.6$ for diiodinated α-Bgt (Hanley et al., 1977), and confirmed with protein determinations according to Lowry et al. (1951), using bovine serum albumin as a secondary standard. The radiopurity of labeled toxins was ascertained by titration of toxin (at concentrations in excess of their apparent K_D) with increasing quantities of *Torpedo* nAChR. At a large excess of receptor over toxin, the proportion of radioactivity sedimenting with receptor-containing membranes serves as a lower limit [due to nonquantitative precipitation of toxin–receptor complexes; see, also, Jones & Thompson (1980)] for the amount of radioisotope associated with toxin and the biological activity of labeled species. Specific activity determinations based on direct counting of radiotoxin aliquots of known concentration were confirmed in toxin titration experiments, where membrane fragment associated specific binding levels at saturation were indexed to the known concentration of toxin binding sites. Over the course of the experiments described herein, the specific activity of the [^{3}H]-α-Bgt preparation used was 12–14 dpm/fmol (5.4–6.4 Ci/mmol). The initial specific activity of the [^{125}I]-α-Bgt preparations varied from 15 to 80 dpm/fmol, and the specific activity of the [$^{125}I_2$]-α-Bgt from the same preparation was twice that of the [^{125}I]-α-Bgt at all times. Decay of the specific activity of the two radioiodinated species exhibited the 60-day half-life of ^{125}I.

Radiotoxin Binding Assays. Rat brain crude mitochondrial fraction membranes were prepared, and toxin binding assays were conducted generally according to Lukas et al. (1979). The crude mitochondrial fraction typically contained approximately 40 fmol of α-toxin/mg of protein. *Torpedo* nAChR-rich membrane fragments were prepared essentially as described by Hazelbauer & Changeux (1974) and contained approximately 100 pmol of α-toxin sites/mg of protein. Binding assays for *Torpedo* membrane fragments were done similarly to those for rat brain membranes, except that toxin–receptor complexes were sedimented at 100000*g* for 30 min in a Beckman 40 rotor. In all data subsequently presented, binding levels are corrected for nonspecific binding (Lukas et al., 1979). For nAChR titration experiments, radiotoxins at concentrations in excess of their apparent K_D were incubated with aliquots of *Torpedo* membranes containing increasing quantities of nAChR. For radiotoxin titration experiments, aliquots of membranes containing nAChR at concentrations less than the apparent K_D were incubated with aliquots of radiotoxin of increasing concentration for either 1 h or overnight (20–24 h). Studies of dissociation of radiotoxin from toxin–receptor complexes followed two general paradigms. After overnight reaction with saturating levels of radiotoxin, and removal of unbound free ligand, samples were either diluted in 10 volumes of buffer (physical dilution) or supplemented with a large (100-fold) excess of unlabeled, native α-Bgt (chemical dilution). In some experiments, samples were supplemented with 1 mM *d*-tubocurarine or carbachol instead of native α-Bgt. Aliquots of each sample were then subjected to centrifugation at different times and assayed for the quantity of radiotoxin–receptor complex remaining. The data presented are typical of those of a large number of experiments.

For toxin association studies, reactions were carried out for specified periods of time until quenched with a large excess of native α-Bgt. Nonradiolabeled toxin competition assays were initiated by addition of an aliquot of membranes to solutions containing a fixed concentration of radiotoxin plus a variable concentration of competing ligand. The concentration of *Torpedo* nAChR, 4 nM, was approximately 8–10 times the concentration of the presumptive nAChR from rat brain. Nonspecific binding contributions to total binding at 10 nM radiotoxin, after 1-h incubation, are for [^{3}H]-α-Bgt, [^{125}I]-α-Bgt, and [$^{125}I_2$]-α-Bgt, respectively, 35%, 40%, and 42% for rat brain and 9%, 5%, and 3% for *Torpedo*. Nonspecific binding increased with time, especially for [$^{125}I_2$]-α-Bgt incubated with rat membranes.

Results

Results of nAChR titration experiments established that the radiopurity of [^{3}H]-α-Bgt and [^{125}I]-α-Bgt exceeds 80% (Figure 1). The data also indicate that the radiopurity of [$^{125}I_2$]-α-Bgt approached that for the two other radiotoxins. An interesting feature of these results is the apparently higher avidity of [^{3}H]-α-Bgt for nAChR relative to radioiodinated toxins. Maximal binding of radiotoxin with receptor is achieved under the given conditions at the lowest nAChR concentration for [^{3}H]-α-Bgt and is not achieved for [$^{125}I_2$]-α-Bgt even at the highest nAChR concentration tested.

Receptor saturation/toxin titration experiments were routinely carried out for 1 h or, in order to more closely approach equilibium conditions, overnight. It should be pointed out that for both short and extended periods of reaction, apparent K_D values are valid relative measures of toxin–receptor affinities (Lukas et al., 1979). Results of a typical series of experiments (Figure 2) illustrate that, in general, observed receptor affinities were highest for [^{3}H]-α-Bgt, and lowest for [$^{125}I_2$]-α-Bgt. An exception was noted with overnight incubation with *Torpedo* membranes, where affinities for [^{125}I]-α-Bgt are about twice those for [^{3}H]-α-Bgt. With the exception of radioiodinated toxin binding to rat brain membranes, apparent affinities are increased on overnight incubation, as the reaction proceeds to equilibium.

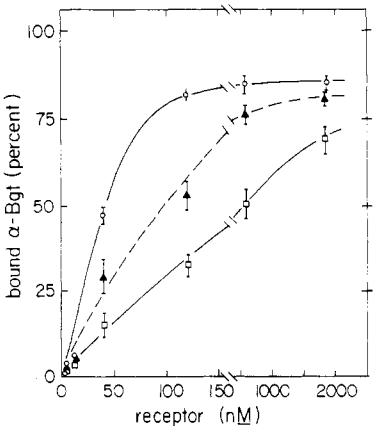

FIGURE 1: Receptor titration curves. After a 2-h incubation, the quantity of radioactivity associated with the membrane pellet (percent of total added radioactivity) is plotted against concentration of *Torpedo* membrane bound nAChR (nM) in order to assess the radiopurity and biological activity of radiolabeled α-Bgt species. The concentration of each toxin was 140 nM in this experiment. (○) [^{3}H]-α-Bgt; (▲) [^{125}I]-α-Bgt; (□) [$^{125}I_2$]-α-Bgt. See Experimental Procedures for assay conditions and design.

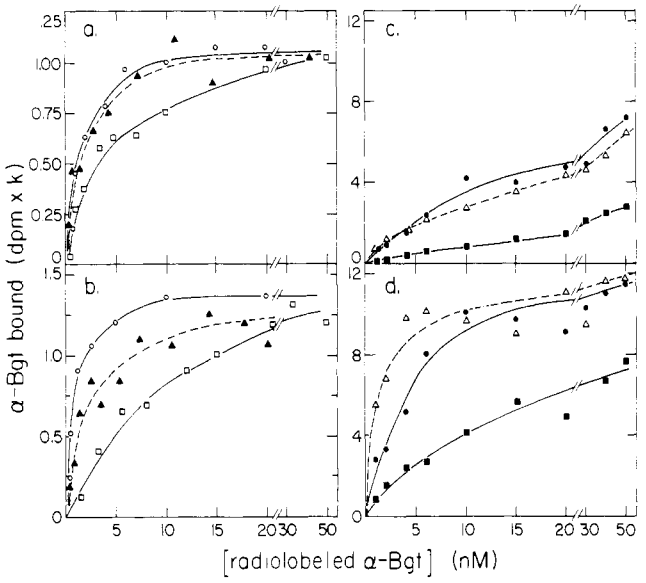

FIGURE 2: Receptor saturation curves. Quantity of radiolabeled toxin specifically bound (dpm × k) to membrane sites at a fixed nAChR concentration is plotted against concentration of radiolabeled toxin (nM). (○; ●) [^{3}H]-α-Bgt; (△; ▲) [^{125}I]-α-Bgt; (□; ■) [$^{125}I_2$]-α-Bgt. (a) Rat brain membranes, 1-h incubation. (b) Rat brain membranes, overnight incubation. (c) *Torpedo* membranes, 1-h incubation. (d) *Torpedo* membranes, 24-h incubation. k values are as follows: for [^{3}H]-α-Bgt, (a–d) $k = 10^{-3}$; for [^{125}I]-α-Bgt, (a and b) $k = 6.25 \times 10^{-5}$ and (c and d) $k = 1.33 \times 10^{-5}$; for [$^{125}I_2$]-α-Bgt, (a and b) $k = 3.12 \times 10^{-5}$ and (c and d) $k = 6.66 \times 10^{-6}$. The concentration of membrane sites from brain was about 0.4 nM and from *Torpedo* was about 3.2 nM in these assays. [^{3}H]-α-Bgt had a specific activity of approximately 11 dpm/fmol and [^{125}I]-α-Bgt approximately 80 dpm/fmol. See Experimental Procedures for further details of the assay conditions and design.

Experiments were also performed to determine association rates of radiotoxins. Typically, membranes were incubated with radiotoxins at final concentrations of 2, 5, 10, 20, 40, and 80 nM, and the period of incubation varied from 10 s to 24 h. Rates of toxin binding were determined from the slope of plots of ln [100(percent sites unoccupied)$^{-1}$] vs. time (Bylund, 1980). The values of these slopes, representing observed association rates, are plotted against radiotoxin concentration in Figure 3. Over the range of radiotoxin concentrations 2–40 nM, these plots are essentially linear, indicating that the apparent association rate constant is nearly independent of toxin

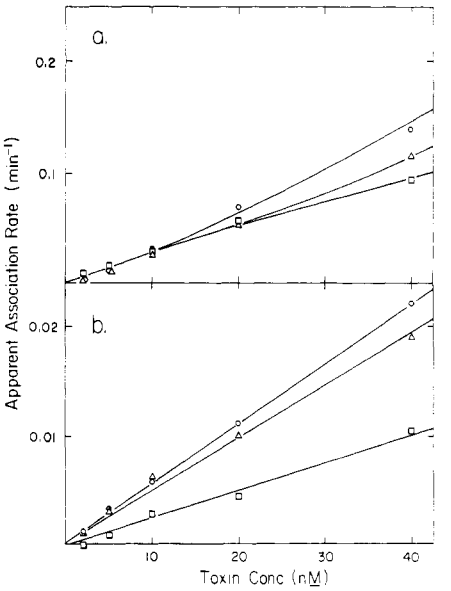

FIGURE 3: Apparent association rate determination. Saturation curves from 2 to 80 nM in toxin concentration were determined as a function of time. Data from the first 10 min were transformed to plot ln [100(percent sites unoccupied)$^{-1}$] against time. Apparent association rates for radiotoxin–receptor interactions (min^{-1}) are calculated and plotted against toxin concentration (nM). (a) Rat brain membranes. (b) *Torpedo* membranes. (○) [^{3}H]-α-Bgt; (△) [^{125}I]-α-Bgt; (□) [$^{125}I_2$]-α-Bgt. Values for k_1 (min^{-1} nM^{-1}) for [^{3}H]-α-Bgt, [^{125}I]-α-Bgt, and [$^{125}I_2$]-α-Bgt are respectively 3.2×10^6, 2.7×10^6, and 2.7×10^6 for rat brain and 5.6×10^5, 5.0×10^5, and 2.5×10^5 for *Torpedo* membranes.

concentration over this range. At 80 nM, the association rates were markedly increased. The increased rates observed at the higher toxin concentrations suggest the possibility of second binding sites with lower affinity. Association rate constants are comparable for binding to rat brain membranes for all three species of radiotoxin. For interactions with *Torpedo* nAChR, rate constants for [^{3}H]-α-Bgt and [^{125}I]-α-Bgt are twice those observed for [$^{125}I_2$]-α-Bgt. In addition, binding to brain of [^{3}H]-α-Bgt and [^{125}I]-α-Bgt is about 6 times more rapid than the binding to *Torpedo* membranes.

In order to further delineate the kinetic properties of radiotoxin–receptor interactions, we determined dissociation rates of toxin from receptor sites. For both rat brain membranes and *Torpedo* membrane bound nAChR, dissociation of receptor–toxin complexes on dilution is characterized by an exponential process: a monophasic decay curve fits the data for [^{3}H]-α-Bgt and a nearly monophasic decay curve fits the data for [^{125}I]-α-Bgt. However, a clearly biphasic dissociation curve was found for [$^{125}I_2$]-α-Bgt (Figure 4). Dissociation of radiotoxin–receptor complexes is accelerated on exposure to a large excess of nonradiolabeled toxin. For dissociation of [^{3}H]-α-Bgt from toxin binding sites, the process is again characterized by a single-exponential decay. Experiments using [^{3}H]-α-Bgt and rat brain membranes indicate that the dissociation rates in the presence of 1 mM carbachol or *d*-tubocurarine are intermediate between those observed under chemical and physical dilution conditions. Another feature of the data is the observation that dissociation of radiotoxin upon chemical dilution follows a biphasic decay process for [^{125}I]-α-Bgt, which is even more pronounced for [$^{125}I_2$]-α-Bgt. The appearance of the biphasic decay process and relative quantities of quickly and slowly dissociating components was found not to be sensitive to the extent of receptor occupation. That is, decay profiles were comparable whether receptor was incubated for 1 h with 4 or 40 nM toxin or overnight with 4 or 6 nM toxin. Independent of the specific conditions used

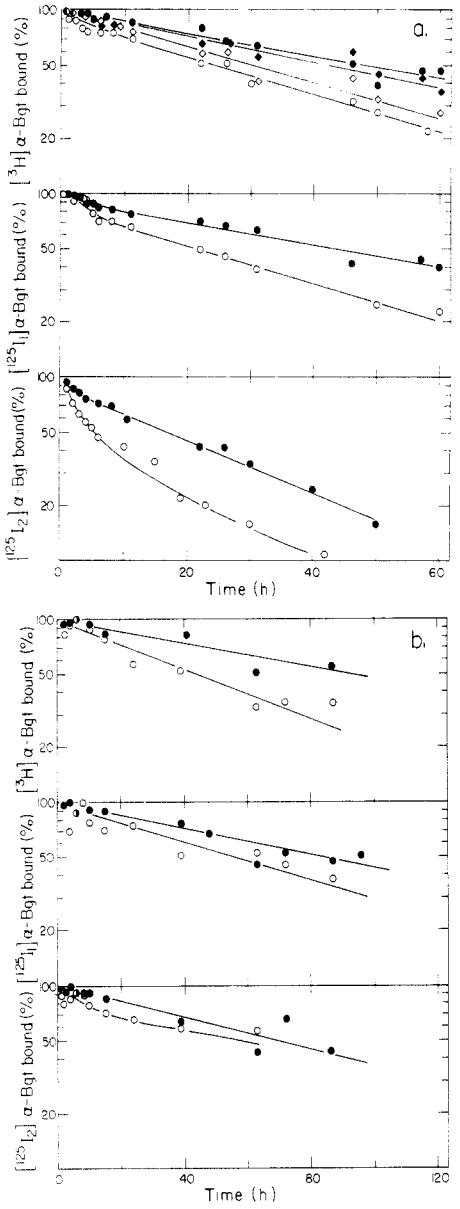

FIGURE 4: Dissociation of radiotoxin–receptor complexes. Extent of receptor site occupation (percent maximal) is plotted against time (h): (a) brain membranes; (b) *Torpedo* membranes. Data shown are for [^{3}H]-α-Bgt (upper panel), [^{125}I]-α-Bgt (middle panel), and [$^{125}I_2$]-α-Bgt (lower panel). Receptor occupancy levels were determined following dilution in the reaction buffer (●) or after addition of excess native α-Bgt [(○) chase]. Data are also shown for dissociation in the presence of 1 mM carbachol (◊) or *d*-tubocurarine (♦). Calculated values for the dissociation rate constant (min^{-1}) from brain membranes are as follows for [^{3}H]-α-Bgt, [^{125}I]-α-Bgt, and [$^{125}I_2$]-α-Bgt, respectively: dilution, 2.36×10^{-4}, 2.69×10^{-4}, 6.24×10^{-4}; chase, 4.62×10^{-4}, 3.86×10^{-4} (80%) and 1.15×10^{-3} (20%), 6.08×10^{-4} (45%) and 2.56×10^{-4} (55%). For [^{3}H]-α-Bgt, $k_{-1} = 3.86 \times 10^{-4}$ (carbachol addition) and 2.75×10^{-4} (*d*-tubocarine addition). Calculated values for the dissociation rate constant (min^{-1}) from *Torpedo* membranes are as follows for [^{3}H]-α-Bgt, [^{125}I]-α-Bgt, and [$^{125}I_2$]-α-Bgt, respectively: dilution, 1.27×10^{-4}, 1.38×10^{-4}, 1.48×10^{-4}; chase, 2.63×10^{-4}, 2.10×10^{-4}, 1.38×10^{-4} (80%) and 7.22×10^{-4} (20%). These dissociation curves are typical of a larger number of dissociation curves for which the $t_{1/2}$ varied depending upon the experimental conditions. However, under comparable conditions the dissociation of toxin from rat brain membranes was always greater than that from *Torpedo* membranes, and for rat brain membranes the rate of dissociation of [$^{125}I_2$]-α-Bgt ≫ [^{125}I]-α-Bgt ≥ [^{3}H]-α-Bgt.

to measure radiotoxin dissociation, the fastest rates are observed for dissociation from rat brain membranes with [$^{125}I_2$]-α-Bgt–receptor complexes; [^{3}H]-α-Bgt–receptor complexes are longest lived. The dissociation rates of the toxins from *Torpedo* membranes were about 50% of the rates from rat brain membranes, and for chemical dilution, [^{3}H]-α-Bgt dissociated more rapidly than [$^{125}I_2$]-α-Bgt.

Table I: Dissociation of α-Bungarotoxin Derivatives from Rat Brain and *Torpedo* Membranes[a]

membrane source	α-toxin	calculated K_D (nM) dilution	chase
brain	[^{3}H]-α-Bgt	0.075	0.14
brain	[^{125}I]-α-Bgt	0.10	0.14 (80)[b] 0.44 (20)
brain	[$^{125}I_2$]-α-Bgt	0.23	0.23 (45) 0.96 (55)
Torpedo	[^{3}H]-α-Bgt	0.23	0.46
Torpedo	[^{125}I]-α-Bgt	0.28	0.42
Torpedo	[$^{125}I_2$]-α-Bgt	0.60	0.56 (80) 2.9 (20)

[a] Calculated K_D values are from the data shown in Figures 2–4. [b] Values in parentheses are percentages.

Table II: Dixon Plot Slopes[a]

nonradiolabeled toxin	radiotoxin [^{3}H]-α-Bgt	[^{125}I]-α-Bgt	[$^{125}I_2$]-α-Bgt
brain			
native α-Bgt	1.02	1.72	2.26
monoiodo-α-Bgt	0.49	0.98	1.52
diiodo-α-Bgt	2.50	1.36	1.36
Torpedo			
native α-Bgt	0.98	1.73	2.25
diiodo-α-Bgt			0.56

[a] The slopes were obtained from competition experiments in which the specific binding of labeled toxin was determined in the presence of increasing ratios (from 0 to 2) of unlabeled toxins. The ratio of b_{max}/b, where b_{max} is the specific (radioactive) binding in the presence of competitor, was plotted vs. the ratio of nonlabeled to labeled toxin.

Experimentally derived values for association and dissociation rate constants are given in the legends to Figures 3 and 4, respectively. The resultant K_D values are summarized in Table I.

In an earlier report, it was suggested that differences in radiotoxin–receptor affinities were evident in binding competition experiments [Lukas(iewicz) et al., 1978]. Data derived from modified Dixon (1953) plots of additional experiments are summarized in Table II. In these transforms, a slope of 1 indicates that radiolabeled and nonradiolabeled toxins are equally effective as ligands interacting at receptor binding sites. Slopes greater than 1 suggest that the receptor has higher affinity for the nonradiolabeled species, while slopes of less than 1 indicate that the receptor has higher affinity for the radiolabeled species. From the results when both brain and *Torpedo* membranes were used, it is evident that native α-Bgt competes most effectively for [$^{125}I_2$]-α-Bgt binding and least effectively for [^{3}H]-α-Bgt. Similar results are obtained when nonradiolabeled, monoiodo-α-Bgt is used as a competing ligand for sites on brain membranes. The results further illustrate that native α-Bgt is the most potent inhibitor of radioiodinated toxin binding. (Anomalous results were obtained with diiodo-α-Bgt vs. [^{3}H]-α-Bgt and diiodo-α-Bgt vs. [$^{125}I_2$]-α-Bgt.)

Discussion

Monoiodinated, diiodinated, and native (tritiated) α-Bgt derivatives, previously shown to be distinguishable on the basis of their ultraviolet and circular dichroism spectra [Lukas-(iewicz) et al., 1978], exhibit characteristic kinetic properties on interaction with nicotinic-type acetylcholine receptors

present in membrane preparations from rat brain and *Torpedo* electric organ. Detailed studies of receptor–radiotoxin binding properties yield quantitatively different values of preequilibrium apparent K_D and association and dissociation rate constants for different radiolabeled α-Bgt derivatives. The interaction of [^{3}H]-α-Bgt with brain and *Torpedo* nAChR fits the simplest receptor binding mechanism, displaying monophasic association (at low toxin concentrations) and dissociation profiles. [^{3}H]-α-Bgt also binds to brain membrane sites with the highest affinity of tested α-Bgt-derived radiotoxins. Affinity of [^{125}I]-α-Bgt for receptor sites is only slightly less than that of [^{3}H]-α-Bgt, except as binding to *Torpedo* nAChR approaches equilibrium. In addition, the dissociation rates for [^{125}I]-α-Bgt–receptor complexes show a small degree of biphasic character. In contrast, affinity of receptor sites for [$^{125}I_2$]-α-Bgt is lowest by all criteria, and decay of [$^{125}I_2$]-α-Bgt–nAChR complexes is clearly biphasic in the presence of excess native α-Bgt.

These results confirm and extend our previous findings [Lukas(iewicz) et al., 1978] and point to the kinetic bases of the observations, replicated herein, that native α-Bgt is the most effective inhibitor of radioiodinated toxin binding and that binding of [^{3}H]-α-Bgt is most resistant to inhibition. In contrast to results of earlier experiments [Lukas(iewicz) et al., 1978] in which a decrease in affinity was not found, the results presented herein document decreased affinity of [$^{125}I_2$]-α-Bgt for nAChR in both rat brain and *Torpedo* electroplax. While differences between binding properties of [^{125}I]-α-Bgt and [^{3}H]-α-Bgt are small, our present evidence again suggests that major alterations in receptor binding activity shown by diiodo-α-Bgt are probably a consequence of alterations in the conformation of the molecule induced by progressive iodination of an exposed tyrosine residue [Lukas(iewicz) et al., 1978]. Provisional results of one experiment suggest that the susceptible residue is Tyr-54 (M. R. Hanley and R. J. Lukas, unpublished experiments), in accordance with the interpretation of Blanchard et al. (1979) regarding iodination of α-Bgt via a nonenzymatic procedure similar to that described herein. In a recent report Wang & Schmidt (1980) have assigned the iodination to the corresponding tyrosine. It is evident, however, that while Tyr-54 is not one of the conserved residues thought to be fundamentally involved in receptor binding (Tsernoglou & Petsko, 1976), it may still influence receptor binding properties in a significant, if not predictable, way (Hanley, 1978).

There are a number of reports in the literature documenting kinetic and equilibrium parameters for reaction of radiolabeled, curaremimetic neurotoxins with nAChR from *Torpedo* (Wang & Schmidt, 1980; James et al., 1980; Blanchard et al., 1979; Weiland et al., 1976; Franklin & Potter, 1972), *Electrophorus* (Weber & Changeux, 1974; Maelicke et al., 1977; Fulpius et al., 1975; Bulger et al., 1977), cat muscle (Barnard et al., 1977), rat muscle (Brockes & Hall, 1975; Kemp et al., 1980), chick muscle (Wang & Schmidt, 1980), and central neural tissues (Wang & Schmidt, 1980; Morley & Kemp, 1981; Lowy et al., 1976; Moore & Brady, 1976; McQuarrie et al., 1976). Direct comparison of those data to the results described in this communication is difficult, however, due to important differences in incubation media, in the use of membrane-bound or solubilized receptor preparations, in the choice of the purified snake toxin used, in the nature of the radiolabeling modification reaction, in the nonoverlapping ranges of toxin and receptor concentrations utilized, and in insufficient documentation of the purity and characteristics of radiotoxin in some cases.

Four studies, in addition to ours, have dealt with differences in binding characteristics caused by labeling of α-Bgt with ^{125}I. Vogel et al. (1972) first noted that [^{125}I]-α-Bgt bound to cultured chick embryonic cells both more rapidly and with as much as a 3-fold lower K_D than did [$^{125}I_2$]-α-Bgt. It appears from data given in their paper that the iodinated toxins contained little of the noniodinated species. Recently, three other reports have appeared characterizing the binding of iodinated derivatives of α-Bgt (Blanchard et al., 1979; James et al., 1980; Wang & Schmidt, 1980). Blanchard et al. used mixtures of [$^{125}I_1$]-α-Bgt and native α-Bgt and concluded that the labeled toxin bound to membrane-bound receptor from *Torpedo* with the same rate constant as the unlabeled toxin. James et al. compared the binding characteristics of nonlabeled toxin, [^{125}I]-α-Bgt, and [$^{125}I_2$]-α-Bgt. They also concluded that monoiodination of the toxin did not modify the binding properties to purified nAChR while [$^{125}I_2$]-α-Bgt bound less rapidly to solubilized nAChR from *T. californica*. Most recently, Wang and Schmidt have compared the binding properties of the diiodinated toxin to those of the monoiodinated toxin and the nonsubstituted toxin. They conclude that diiodination reduced the binding rate by a factor of 2–3 but did not change the dissociation rate ($t_{1/2} \sim 3$ h) from the receptor obtained from chick optic lobes. No differences were reported between the association rates of native toxin and the monoiodinated toxin. (It should be pointed out, however, that the chromatographic step to purify labeled toxins was carried out at pH 7.4 and probably a mixture of the monoiodinated and noniodinated toxin has been used in two of these recent studies.) Thus, monoiodination of α-Bgt Tyr-54 is a relatively innocuous procedure. This can be contrasted with the labeling of α-Bgt with ^{3}H-labeled pyridoximine phosphate which leads to a 9-fold decrease in receptor binding (James & Thompson, 1980). We would agree with the conclusion that [$^{125}I_2$]-α-Bgt differs significantly from the native toxin and [^{125}I]-α-Bgt and that detailed kinetic studies of the binding of iodinated α-Bgt should not be made with mixtures of the two nonidentical forms. It is also evident that contrary to some accounts in the literature [e.g., Blanchard et al. (1979)], the dissociation of toxin–membrane nAChR complexes does have a measurable half-life, and toxin–receptor interactions consequently have measurable dissociation constants.

Several other observations warrant specific comment. The acceleration of decay of radiotoxin–nAChR complexes on exposure to excess native α-Bgt may have a simple explanation, based on the assumption that the overall receptor–toxin interaction is comprised of numerous ionic, hydrophobic, and van der Waals contacts. In the absence of added nonradiolabeled ligand, the probability that enough receptor–toxin contacts will be simultaneously broken in the presence of solvent and solute molecules, with low affinity and specificity for those sites, will be small. Competing ligand, however, possesses sufficient affinity and specificity for those sites that the probability of simultaneous contact blockade is increased, particularly when the ligand shares specific contacts with radiotoxin. The intermediate ability of *d*-tubocurarine to accelerate dissociation of bound [^{3}H]-α-Bgt from rat brain membranes suggests that it shares fewer contact points with [^{3}H]-α-Bgt than does native α-Bgt. The ability of carbachol to induce toxin dissociation may also reflect contributions due to carbachol-induced alterations in receptor state. The explanation of toxin binding inhibition results advanced in a previous communication [Lukas(iewicz) et al., 1978], which may explain decreased affinity of nAChR for [$^{125}I_2$]-α-Bgt as described herein, based on steric fit considerations and for-

mation of ternary toxin–receptor–toxin complexes is also consistent in the context of the present results. It is, however, curious that some logical expectations of such a toxin binding model are not borne out by the present data—such as the absence of clear 1:1 stoichiometries for slowly and rapidly dissociating [$^{125}I_2$]-α-Bgt–nAChR complexes under chase conditions. Finally, the documented differences in receptor–toxin complex dissociation properties for [^{3}H]-α-Bgt and [$^{125}I_2$]-α-Bgt are subject to alternative explanations and prospecti. On the one hand, one might argue that evaluation of receptor–diiodotoxin interactions should be treated cautiously, in that anomalous binding behavior may be attributed to the behavior of the derivative itself. On the other hand, use of iodinated toxins might be useful in revealing microheterogeneities of receptor sites or toxin binding mechanisms.

References

Barnard, E. A., Coates, V., Dolly, J. O., & Mallick, B. (1977) *Cell Biol. Int. Rep. 1*, 99.

Bennett, E. L., Morimoto, H., Hanley, H. R., & Lukas, R. J. (1979) *Soc. Neurosci. Abstr. 5*, 397.

Blanchard, S. G., Quast, U., Reed, K., Lee, T., Schimerlik, M. I., Vandlen, R., Claudio, T., Strader, C. D., Moore, H. P. H., & Raftery, M. A. (1979) *Biochemistry 18*, 1875.

Brockes, J. P., & Hall, Z. W. (1975) *Biochemistry 14*, 2092.

Bulger, J. E., Fu, J.-J. L., Hindy, E. F., Silberstein, R. L., & Hess, G. P. (1977) *Biochemistry 16*, 684.

Byland, D. B. (1980) in *Receptor Binding Techniques, 1980 Short Course Syllabus*, p 70, Society for Neuroscience, Bethesda, MD.

Dixon, M. (1953) *Biochem. J. 55*, 170.

Eterović, V. A., Aune, R. G., & Bennett, E. L. (1975a) *Anal. Biochem. 68*, 394.

Eterović, V. A., Hebert, M. S., Hanley, M. R., & Bennett, E. L. (1975b) *Toxicon 13*, 37.

Franklin, G. I., & Potter, L. T. (1972) *FEBS Lett. 28*, 101.

Fulpius, B. W., Maelicke, A., Klett, R., & Reich, E. (1975) in *Cholinergic Mechanisms* (Waser, P. G., Ed.) p 373, Raven Press, New York.

Hanley, M. R. (1978) Doctoral Thesis, University of California, Berkeley, CA.

Hanley, M. R., Eterović, V. A., Hawkes, S. P., Hebert, A. J., & Bennett, E. L. (1977) *Biochemistry 16*, 5840.

Hazelbauer, G. L., & Changeux, J.-P. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1479.

Heidmann, T., & Changeux, J.-P. (1978) *Annu. Rev. Biochem. 47*, 317.

James, R. W., Bersinger, N. A., Schwendimann, B., & Fulpius, B. W. (1980) *Hoppe-Seyler's Z. Physiol. Chem. 361*, 1517.

Jones, S. W., & Thompson, W. R. (1980) *Anal. Biochem. 101*, 261.

Kemp, G., Morley, B., Dwyer, D., & Bradley, R. J. (1980) *Membr. Biochem. 3*, 229.

Lee, C. Y. (1971) in *Neuropoisons* (Simpson, L. L., Ed.) Vol. 1, p 21, Plenum Press, New York.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Lowy, J., McGregor, J., Rosenstone, J., & Schmidt, J. (1976) *Biochemistry 15*, 1522.

Lukas, R. J., Morimoto, H., & Bennett, E. L. (1979) *Biochemistry 18*, 2384.

Lukas(iewicz), R. J., Hanley, M. R., & Bennett, E. L. (1978) *Biochemistry 17*, 2308.

Maelicke, A., Fulpius, B. W., Klett, R. P., & Reich, E. (1977) *J. Biol. Chem. 252*, 4811.

McQuarrie, C., Salvaterra, P. M., deBlas, A., Routes, J., & Mahler, M. R. (1976) *J. Biol. Chem. 251*, 6335.

Moore, W. M., & Brady, R. N. (1976) *Biochim. Biophys. Acta 444*, 252.

Morley, B. J., & Kemp, G. E. (1981) *Brain Res. Rev. 3*, 81.

Tsernoglou, D., & Petsko, G. A. (1976) *FEBS Lett. 68*, 1.

Tu, A. T. (1977) *Venoms: Chemistry and Molecular Biology*, Wiley, New York.

Vogel, Z., Sytkowski, A. J., & Nirenberg, M. W. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3180.

Wang, J.-K., & Schmidt, J. (1980) *J. Biol. Chem. 255*, 11156.

Weber, M., & Changeux, J.-P. (1974) *Mol. Pharmacol. 10*, 1.

Weiland, G., Georgia, B., Wee, V. T., Chignell, C., & Taylor, P. (1976) *Mol. Pharmacol. 12*, 1091.